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Taurine 9

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Taurine 9

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It is with great sadness that the Editors of this Proceedings relate to the authors and the scientific community that one of the true leaders in the taurine field, Dr. Junichi Azuma, passed away on September 20, 2014. He had suffered from bladder cancer for 6 years. During that time, he still engaged in research and in educational activities at his University. Junichi is survived by his wife, Kimiko, and three children (Junya, Risa, and Junzi).

At the time of his death, Dr. Azuma was professor of Hyogo University of Health Sciences, Faculty of Pharmacy, and emeritus professor of Osaka University. He was an internationally recognized researcher who completed over 300 clinical trials and published over 500 papers in the field of pharmacogenomics and clinical pharmacology. He is best known for his pioneer work in the taurine field. After showing that taurine was extremely effective in preventing severe cardiac damage in animal models of heart failure, he performed clinical trials on patients suffering from

congestive heart failure. His first heart failure patient that received taurine therapy showed significant improvements in the signs of heart failure (fatigue, orthopnea, and dyspnea). Prior to being treated with taurine, the patient was being treated with furosemide, digoxin, and spironolactone, but taurine therapy reduced the need for digoxin and spironolactone and the classification of severity fell from NY class IV to NY class II. Dr. Azuma continued with his clinical trials and taurine was approved for the treatment of heart failure in Japan. After achieving this major goal, he began examining the mechanism underlying the beneficial effects of taurine and discovered that taurine partially prevents ventricular remodeling by antagonizing the actions of angiotensin II and the catecholamines, attenuating calcium overload, and suppressing apoptosis. More recently, Junichi developed a mouse model of taurine deficiency, which is characterized by muscle weakness, retinal degeneration, development of a cardiomyopathy, and premature aging. Recently, his research has focused on the effect of taurine deficiency on senescence.

Dr. Azuma hosted two international taurine meetings, a 1995 Osaka meeting and a 2007 Shimoda meeting. One of his last projects was to establish a Japanese taurine society. Clearly, Dr. Azuma's life was devoted to the promotion of taurine research. He will be sorely missed by the taurine community.

Preface

The Nineteenth International Taurine Meeting "Taurine—a *very essential* amino acid" was held on May 21–24, 2014, in Kraków, Poland. The Department of Immunology, Jagiellonian University Medical College, was honored as the host and organizer of the congress. This biannual congress was held in Poland for the first time in the congress's history. Importantly, it took place in a special year for our University. The Jagiellonian University was founded 650 years ago and is presently one of the oldest universities in Central Europe. We are very proud that our conference was included in the other jubilee solemnities of the University.

Approximately 120 participants from 17 countries, including young scientists as well as experts in taurine research, attended the meeting. We are very pleased that scientists from new countries presented their achievements. It shows how interest in taurine research has been growing. A total of 109 papers were presented as either oral or poster presentations. The scientific program consisted of six sessions, with topics ranging from the physiological role of taurine in maintaining human body homeostasis to the amino acid's antioxidant properties as well as the therapeutic actions of taurine derivatives on cardiovascular, nervous, inflammatory, and endocrine diseases. Also presented were novel animal experimental models that are proving useful in testing the physiological functions and metabolism of the sulfur amino acids, including taurine.

On behalf of the Organizing Committee we would like to thank Taisho Pharmaceutical Co., Ltd., Tokyo, Japan, and Faculty of Medicine of Jagiellonian University Medical College (Leading National Research Centre 2012–2017) for their generous financial support. We owe a special gratitude to chairmen and members of the Scientific Committee (Dr. Russell Chesney, Dr. Abdeslem El Idrisi, Dr. Simo Oja) for their effort in construction of the scientific program of the Conference and reviewing the proceedings published in *Taurine 9*. In addition, we would like to thank all participants of the meeting and the authors of the papers in this volume.

Finally, we would like to express our great appreciation for the outstanding effort before, during, and after the conference of Dr. Marta Ciszek-Lenda and Maria Walczewska, who helped to ensure the success of the meeting.

We are pleased to provide you with *Taurine 9*, which contains the proceedings of the Nineteenth International Taurine Meeting consisting of 73 papers accepted by the reviewers.

Krakow, Poland Janusz Marcinkiewicz
Mobile, AL Stephen W. Schaffer

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Part I
Biology of Taurine and Related Sulfur-Amino Acids

A Novel Cysteine Sulfinic Acid Decarboxylase Knock-Out Mouse: Comparison Between Newborn and Weanling Mice

Eunkyue Park, Seung Yong Park, Carl Dobkin, and Georgia Schuller-Levis

Abbreviations

CSAD	Cysteine sulfinic acid decarboxylase
CSAD KO	Cysteine sulfinic acid decarboxylase knockout mice
CDO	Cysteine dioxygenase
CDO KO	Cysteine dioxygenase knockout mice
ADO	Cysteamine (2-aminoenthanethiol) dioxygenase
WT	Wild type (CSAD+/+)
HT	Heterozygotic mice (CSAD+/−)
HO	Homozygotic mice (CSAD−/−)
HOT	Homozygotic mice treated with 0.05 % taurine
TauT	Taurine transporter
TauT KO	Taurine transporter knockout mice
Gpx 1	Glutathione peroxidase 1
Gpx 3	Glutathione peroxidase 3
Prdx 2	Peroxireductase 2
Prdx 3	Peroxireductase 3
Prlr	Prolactin receptor
Ltf	Lactoferrin

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Upp 2	Uridine phosphorylase 2
Sds	Serine dehydratase
G1 G2, G3, G4	Generation 1, 2, 3 and 4
G1 HO	HO (CSAD$^{-/-}$) mice born from HT (CSAD$^{+/-}$) parents
G2 or G3 HO	Mice born from G1 HO or G2 HO parents

1 Introduction

The essential physiological functions of taurine in development of the brain and eye, in reproduction, endocrine regulation, kidney and cardiovascular function, membrane stabilization, osmotic regulation as well as immune function have been well documented in many laboratories (Schuller-Levis and Park 2006; Sturman 1993). Four gene products are critical to taurine homeostasis: cysteine dioxygenase (CDO; EC 1.13.11) oxidizes cysteine to cysteine sulfinic acid which is converted to hypotaurine which is then oxidized to taurine (Stipanuk 2004; Bella et al. 2000; Hosokawa et al. 1990); cysteamine dioxygenase ADO (EC1.13.11.19) which is converted cysteamine to hypotaurine (Dominy et al. 2007); the taurine transporter (TauT) (Uchida et al. 1992); and cysteine sulfinic acid decarboxylase, CSAD (EC 4.1.1.29), which is the enzyme that converts cysteine sulfinic acid to hypotaurine (Park et al. 2002).

Taurine is considered a conditionally essential amino acid in humans and primates and is required during their development. Taurine deficient animals are useful in investigations of taurine's physiological functions because it is possible to study both taurine deficiency itself and the effect of supplementation with taurine in food or drinking water. Cats and rodents have been used as animal models for taurine studies because their taurine levels could be easily manipulated (Sturman 1993; Sturman and Messing 1991, 1992). Cats have been used for taurine studies because they produce only low levels of CDO and CSAD leading to a dependence on dietary sources of taurine. However, the cat model has limitations including a long gestation period, a heterogeneous genetic background and a relatively large maintenance expense and the absence of genetic, molecular and immunological reagents. Rodents have high levels of CSAD (Schuller-Levis and Park 2006; Sturman 1993; Huxtable 2000) and taurine is not essential to their diet. Due to high concentrations of taurine in rodents, competitive inhibitors of taurine transport including guanidinoethanesulfonate (GES) or β-alanine have been used to produce taurine deficiency (Bonhaous et al. 1985; Dela Rosa and Stipanuk 1984; Jong et al. 2010). However, these chemicals have toxic side effects.

Recently genetically modified mice have been developed to model taurine deficiency using gene targeting methods. These taurine-deficient knock-out mice include taurine transporter knockout mice (TauT KO) and cysteine dioxygenase knockout mice (CDO KO). Two TauT KO mouse models show reduced levels of taurine in various tissues including heart, brain, muscle, kidney and liver (Heller-Stilb et al. 2002; Warsulat et al. 2007; Ito et al. 2008). These TauT KO models demonstrate developmental effects in various organs including the retina, liver,

brain, muscle and heart. A cysteine dioxygenase deficient (CDO KO) model was produced by deleting CDO, thereby disabling the production of cysteine sulfinic acid, a substrate for CSAD, from cysteine (Ueki et al. 2011, 2012; Roman et al. 2013). These mice have severe taurine deficiency and increased catabolism of cysteine to hydrogen sulfide, which leads to pulmonary and pancreatic toxicity.

Recently, our laboratory produced a cysteine sulfinic acid decarboxylase knockout mouse (CSAD KO) as a novel mouse model of taurine deficiency (Park et al. 2014). CSAD is an enzyme for taurine biosynthesis and a cytosolic enzyme expressed primarily in liver and kidney (Sturman 1993; Park et al. 2002). The level of CSAD activity determines the need for dietary taurine. Our laboratory demonstrated high neonatal mortality in the third and fourth generation of $CSAD^{-/-}$ homozygotes (G3 HO and G4 HO) and restoration of neonatal survival by the addition of 0.05 % taurine added to the drinking water. Compared to wild type (WT), taurine concentrations in the liver and brains of newborn pups are significantly lower except in G1 HO, which are born from $CSAD^{+/-}$ heterozygous (HT) dams which have near normal levels of serum taurine. Low taurine concentrations in the liver and brain in HOs are significantly restored by supplementation of taurine in the drinking water of the dam. Gene expression of prolactin receptor (Prlr) and lactoferrin (ltf) is decreased but gene expression of glutathione peroxidase 3 (Gpx 3) and peroxireductase (Prx 2) increased, suggesting oxidative stress may be involved in neonatal mortality. Here we compare these phenotypic changes in newborn pups and weanling mice.

2 Materials and Methods

2.1 Materials

Chemicals used in this study were purchased from Sigma Chemicals (St. Louise, MO) if not otherwise noted. Oligonucleotide primers for PCR for genotype were obtained from Eurofins MWG Operon (Huntsville, AL). Primers were designed by Primer Designer 4 (Scientific and Educational Software, Cary, NC). Taq polymerase and deoxynucleotides were purchased from New England Biolabs (Ipswich, MA). Agarose was obtained from Lonza Group LTD (Rockland, ME). Trizol and RNeasy kit for RNA extraction were obtained from Invitrogen and Qiagen (Valencia, CA), respectively. The SYBR master mix and primers used in RT^2 qPCR were purchased from Qiagen.

2.2 CSAD KO Mice

CSAD KO mice were produced previously described from our laboratory (Park et al. 2014). Briefly, chimeric CSAD KO mice were produced by injection of cells from a gene trap ES cell line (XP0392) into C57BL/6 (B6) blastocysts at the

Mouse Mutant Regional Resource Centers (MMRRC, UC Davis, CA) which were implanted into a pseudopregnant B6 mouse. Chimeric mice from MMRRC were mated with B6 (Jackson Laboratories, Bar Harbor, ME) in the animal colony at NYS Institute for Basic Research in Developmental Disabilities. Heterozygous siblings were mated to produce $CSAD^{-/-}$ homozygous pups (HO). Experimental mice were fed taurine-free chow (LabDiet^R, PMI Nutrition International, St. Louis, MO). Taurine concentrations in commercial food were confirmed by HPLC. All mice were kept under 12-h day/night with free access to food and water. Some experimental animals had exogenous taurine added to their water at 0.05 % as indicated in Results. For optimum reproductive performance, one or two females were mated to a single male. Both females and males used for mating in the taurine-treated groups were supplemented with taurine. Animals were weaned at 3 weeks of age. All mice at 1 M used in this study were separated from their dam for 1 week. All procedures involving live animals were approved by the Institutional Animal Care and Use Committee of IBR.

2.3 High Performance Liquid Chromatography (HPLC)

The liver and brain as well as plasma were obtained from all groups including WT and HT as well as G1 HO, G2 HO, G3 HO and G4 HO at birth (PD1) and 1 month of age (1M) after mice were injected *ip* with avertine (250 mg/Kg). These samples were also collected from the taurine-treated groups including G2 HOT, G3 HOT and G4 HOT. Taurine concentrations were determined using HPLC (Waters, Milford, MA) (Battaglia et al. 1999). Briefly, tissues and plasma were homogenized using 5 % TCA and centrifuged for removal of proteins. After samples were dried using a Speedvac (Savant, Holbrook, NY), they were derivatized using phenylisothiocyanate (PITC) and separated using a C18 column with a gradient of acetate buffer containing 2.5 % acetonitrile (pH 6.5) and 45 % acetonitrile solution containing 15 % methanol at 45 °C. The flow rate was 1 ml/min. Taurine concentrations were determined by comparison to a standard.

2.4 RT^2 qPCR Analysis

Total RNA was extracted using RNeasy kit (Qiagen) from the liver and was reverse-transcribed using cDNA kit according to the manufacturer's instruction (Qiagen). Quantitative real time PCR with 10 ng of cDNA were carried out in duplicate in a 7300 real-time PCR system (Eppendorff, Hauppauge, NY) using the SYBR master mix (Qiagen) and the following cycles: 2 min at 50 °C, 10 min at 95 °C and 40 cycles each at 95 °C for 15 s and 60 °C for 60 s (Park et al. 2014). RT^2 qPCR analysis was also carried out according to manufacturer's manual using β-actin as a control. All primers were purchased from Qiagen. For data analysis the Ct method

was used; for each gene fold-changes were calculated as difference in gene expression of G3 HO and G3 HOT, compared to that in WT. ΔCt was calculated by subtraction of Ct of β-actin from Ct of the interesting gene. ΔΔCt was calculated by subtraction of ΔCt of WT from ΔCt of G3 HO or H3 HOT. Fold change was determined by $2^{(-\Delta\Delta Ct)}$. More than 1 indicates gene up-regulation and less than 1 indicates gene down-regulation.

2.5 Statistical Analysis

Data are presented as mean ± SE. Statistical significance was determined using Statistica 8 (StatSoft, Tulsa, OK). Significant differences between groups were determined as $p < 0.05$ using LSD or Tukey HSD in post-hoc under one way ANOVA or t-test.

3 Results

3.1 Taurine Concentrations in the Brain, Liver and Plasma at 1 Month of Age in CSAD KO

We used HPLC to measure taurine concentrations in the liver and brain as well as plasma because the liver is a major organ for taurine production by CSAD while the brain requires taurine. Plasma taurine was measured because the vascular system supplies the taurine required by various organs.

Inactivation of one CSAD allele did affect taurine levels at 1 M: Although brain and plasma taurine concentrations in $CSAD^{+/-}$ were not decreased significantly, liver taurine concentrations were decreased to 77 % of wild type (WT). Previously, we demonstrated that G1 $CSAD^{-/-}$ born from $CSAD^{+/-}$ dams show higher taurine concentrations in the liver and brain compared to G2, G3 and G4 $CSAD^{-/-}$, presumably because of taurine transported through the placenta (Grillo et al. 2008). However, taurine concentrations in the liver, brain and plasma of G1 $CSAD^{-/-}$ at 1 M of age (1 week after weaning) fell to the same levels as in G2, G3 and G4 $CSAD^{-/-}$ (Figs. 1, 2 and 3).

While taurine concentrations in the liver from all generations of $CSAD^{-/-}$ were decreased to approximately 5 % of WT level at 1 M, taurine concentrations in the brain of $CSAD^{-/-}$ decreased to 40 % of WT. Supplementation with 0.05 % taurine in the drinking water brought liver and brain taurine concentrations to 10 and 76 % of wild type (WT), respectively. Thus, while brain taurine concentrations in $CSAD^{-/-}$ treated with taurine (HOT) were significantly increased, liver taurine concentrations were not. At PD1, G3 and G4 HOT taurine concentrations were significantly increased in both brain and liver.

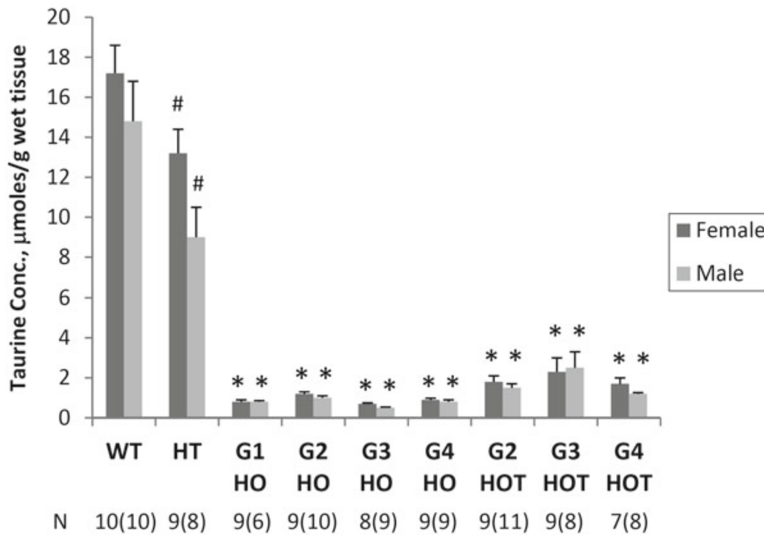

Fig. 1 Taurine concentrations in the liver at 1 M. The livers from designated number of female and male mice were homogenized with 5 % TCA and centrifuged at 10,000 g for 20 min. The supernatants were collected and dried using a Speedvac. Dried samples were derivatized using PITC. PITC-labeled taurine was determined using HPLC. Data are expressed as µmol/g wet tissue weight, mean ± SE in the liver. The number of females and males is shown in the figure. *Parentheses* are males. *$p < 0.0002$ and #$p < 0.002$ statistically significant, compared to WT

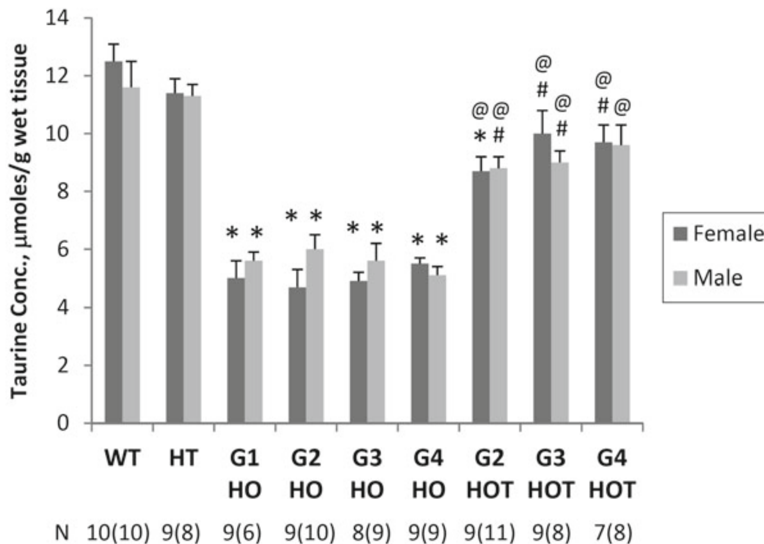

Fig. 2 Taurine concentrations in the brain at 1 M. Brain samples from 1 M mice were analyzed as in Fig. 1. Data are expressed as µmol/g wet tissue weight, mean ± SE in the brain. The number of females and males is shown in the figure with males in *parentheses*. *$p < 0.002$ and #$p < 0.05$ statistically significant, compared to WT. @$p < 0.01$ statistically significant, compared to G2, G3 and G4 HO, respectively

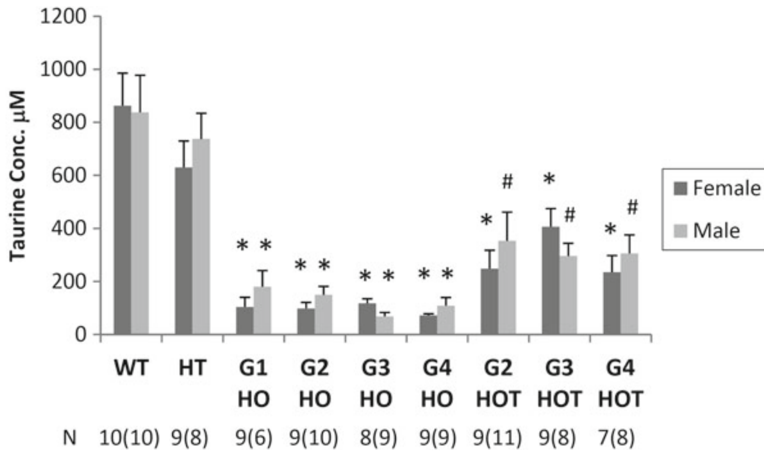

Fig. 3 Plasma taurine concentrations at 1 M. The plasma samples from 1 M mice were analyzed as in Fig. 1. Data are expressed as µM, mean ± SE. The number of females and males is shown in the figure with males in *parentheses*. *$p < 0.0002$ and #$p < 0.002$ statistically significant, compared to WT. Taurine level in treated (HOT) mice is significantly increased over the level in untreated (HO) mice ($F = 50.111$, $p < 0.001$)

Plasma concentrations in all generations of $CSAD^{-/-}$ were decreased to less than 14 % of WT and were restored to 65 % of WT with taurine supplementation in the drinking water compared to WT (Fig. 3). Females and males in all groups were not significantly different in taurine concentrations in brain, liver or plasma.

3.2 *Gene Expression Measured Using RT² qPCR*

We examined gene expression in CSAD KO liver using a microarray analysis. This specifically focused on genes that were candidates for influencing neonatal mortality, genes related to taurine metabolism and transport, genes for anti-oxidant enzymes, nucleotide and amino acid metabolic enzymes as well as for lactoferrin and the prolactin receptor. Taurine-related genes included CSAD, CDO, cysteamine dioxygenase (ADO) and TauT (Table 1). In contrast to gene expression at PD1, CDO was significantly decreased and TauT was significantly increased more than twofold at 1 M in G3 HO. CDO was restored in G3 HOT whereas TauT was not significantly different in G3 HOT compared to G3 HO. As expected, no CSAD expression was detected at PD1 and 1 M. ADO at both PD1 and 1 M was unaffected in both G3 HO and G3 HOT compared to WT.

Since taurine is an antioxidant we examined the expression of genes for antioxidant enzymes included glutathione peroxidase 1 and 3 (Gpx 2 and 3) as well as peroxireductase 2 (Prdx 2) (Table 2). Gpx 3 in G3 HO at 1 M was significantly increased more than twice that of WT just as it had been at PD1. However, Gpx 1

Table 1 Fold change of taurine-related genes in G3 HO and G3 HOT in the liver compared to WT

Gene	Genotype	PD1	1 month
Csad	WT	1.0 ± 0.03[a]	1.0 ± 0.1[b]
	G3 HO	<0.002**	<0.01**
	G3 HOT	<0.001**	<0.01**
Cdo	WT	1.0 ± 0.05	1.0 ± 0.06
	G3 HO	1.3 ± 0.10*	0.7 ± 0.03*
	G3 HOT	1.1 ± 0.09	0.8 ± 0.1
Ado	WT	1.0 ± 0.03	1.0 ± 0.05
	G3 HO	1.1 ± 0.06	1.3 ± 0.3
	G3 HOT	1.3 ± 0.08*	1.1 ± 0.2
TauT	WT	1.0 ± 0.06	1.0 ± 0.05
	G3 HO	1.2 ± 0.07	2.0 ± 0.2**
	G3 HOT	1.2 ± 0.09	2.4 ± 0.2**

*Significantly different compared to WT, $p < 0.05$
**Significantly different compared to WT, $p < 0.001$
[a]Data represent mean ± SE from four WT, six G3 HO and four G3 HOT at PD1, respectively
[b]Data represent mean ± SE from four mice in each group

Table 2 Fold change of antioxidant genes in G3 HO and G3 HOT in the liver compared to WT

Gene	Genotype	PD1	1 month
Gpx1	WT	1.0 ± 0.05	1.0 ± 0.09
	G3 HO	1.5 ± 0.11[a]	1.4 ± 0.1
	G3 HOT	1.2 ± 0.09	1.6 ± 0.2*
Gpx3	WT	1.0 ± 0.14	1.0 ± 0.07
	G3 HO	2.5 ± 0.51**	2.0 ± 0.3**
	G3 HOT	0.9 ± 0.08	0.8 ± 0.1
Prdx2	WT	1.0 ± 0.05	1.0 ± 0.07
	G3 HO	1.6 ± 0.34	1.1 ± 0.2
	G3 HOT	1.0 ± 0.05	0.9 ± 0.1
Prdx3	WT	1.0 ± 0.10	ND
	G3 HO	1.1 ± 0.08	
	G3 HOT	1.0 ± 0.07	

*Significantly different, $p < 0.05$. **$p < 0.001$. At PD1, four mice were used in WT and G3 HOT and six mice were used in G3 HO. At 1 month, four mice were used in all groups
[a]Data are expressed as mean ± SE

and Prdx 2 in G3 HO were not changed at 1 M even though Gpx 1 had been increased at PD1. Taurine supplementation restored Gpx 3 in G3 HOT to WT level at 1 M. While both uridine dephosphorylase 2 (Upp 2), a nucleotide metabolic enzyme, and serine hydratase (Sds), a amino acid metabolic enzyme, in G3 HO at PD1 were increased significantly, Upp 2 and Sds in G3 HO at 1 M were not different compared

Table 3 Gene expression of Upp2 and Sds at PD1 and 1 month in the liver

	Genotype	PD1	1 month
Upp2	WT	1.0 ± 0.02[a]	1.0 ± 0.08
	G3 HO	1.9 ± 0.51	0.8 ± 0.08
	G3 HOT	0.9 ± 0.10	0.7 ± 0.09*
Sds	WT	1.0 ± 0.22	1.0 ± 0.1
	G3 HO	2.3 ± 0.42*	0.8 ± 0.09
	G3 HOT	1.2 ± 0.10	0.6 ± 0.1*

*Significantly different, $p < 0.05$ compared to WT
[a]Data represent mean ± SE. Four mice were used in WT, G3 HO and G3 HOT at PD1 and 1 month

to WT (Table 3). Taurine treatment decreased Upp2 and Sds expression significantly in G3 HOT. Ltf and Prlr were significantly decreased in G3 HO at 1 M, as they had been a PD1. However, taurine treatment restored both at 1 M while only Prlr was restored at PD1.

4 Discussion

Weanling CSAD KO at 1 M showed a profile of taurine distribution in the liver and brain (Fig. 1, 2, and 3) that was different from the profile at PD1 (Park et al. 2014). At PD1, taurine concentrations in both the brain and liver from G2 and G3 HO were approximately 80 % lower than WT. Taurine-treated G2 and G3 HO dams had pups with PD1 liver and brain taurine concentrations that were substantially and similarly increased. However, at 1 M, taurine concentrations in the HO brain were a higher percentage of WT than in the liver (Figs. 1 and 2). After taurine treatment, CSAD−/− liver taurine was significantly less at 1 M than at PD1 despite the increase in liver TauT gene expression (Table 1). In contrast treatment substantially restored taurine levels in the brain at 1 M. Plasma taurine was increased significantly ($p < 0.001$) to approximately 65 % of WT level in the taurine-supplemented CSAD−/− mice (Fig. 3). Taurine concentrations were significantly increased in the brain of these mice but not in the liver which suggests that because the brain is not a major biosynthetic source of taurine, it may be better equipped to utilize the taurine supplied though the drinking water than the liver is. This suggests that when taurine is required for physiological function, taurine may be actively transported from the circulatory system to the organ(s) via TauT. Taurine concentrations in the splenocytes from G3 HO were decreased 56 % compared to WT, similar to the brain (data not published).

These results were similar to those in taurine-deficient knockout mouse models and in cats (Sturman 1993). Taurine concentrations in the liver from CDO KO and TauT KO are significantly decreased (Heller-Stilb et al. 2002; Ito et al. 2008; Ueki et al. 2011). However, in our study taurine concentrations in the liver and brains from females and males in all groups were not significantly different although

taurine concentrations in CDO KO females were higher than in males. Physical phenotypes observed in CDO KO (excess lacrimation and partial palpebral closure, plantigrade stance, hyperextensible toes and less-erect ears) were not observed in CSAD KO. Although neonatal mortality was detected in G3 and G4 HO, surviving CSAD KO grew normally without weight loss compared to WT. However, CDO KO weight and size is less than WT. Taurine deficiency in the cat is similar to CSAD KO with respect to taurine levels. Taurine concentrations in the liver from adult cats without taurine treatment were remarkably decreased compared to adult cats fed 0.05 % taurine. However, the developmental defects in surviving offspring from female taurine-deficient cats such as hind leg development and a peculiar gait characterized by excessive abduction and paresis, were not detected in CSAD KO (Sturman 1993).

CSAD−/− gene expression at 1 M is also unlike PD1. CDO in the liver from G3 HO was significantly increased due to feedback inhibition (accumulation of CSAD in CSAD KO) (Table 1). TauT expression in the liver of G3 HO and G3 HOT was also increased compared to WT at 1 M but not at PD1, indicating TauT may facilitate taurine transport to compensate for decreased taurine concentrations. CSAD gene expression was absent in both groups at both PD1 and 1 M, confirming deletion of the CSAD. The antioxidant enzyme, Gpx 3 expression was increased at 1 M although both Gpx 1 and 3 expression were increased at PD1 (Schaffer et al. 2009; Brigelius-Flohe and Maiorino 2013; Lubos et al. 2011). Alteration in these genes may contribute to neonatal death at PD1. Although G3 HO thrived, antioxidant enzymes may be needed for survival because mice are under oxidative stress due to taurine deficiency. Gpx 3 expression was restored in G3 HOT suggesting taurine may be an antioxidant. Since newborn G3 CSAD−/− often lacked milk spots (MS), a predictor of survival in newborn mice, we examined the gene expression of Ltf and Prlr. Ltf has innate immune function to protect newborn offspring from infection and is elevated in colostrum (Ward and conneely 2004; Legrand and Mazurier 2010; Legrand 2012). Ltf is widely present in fluids such as milk and colostrum with a high affinity for iron. Lft, an important component of the innate immune system, is important in bacteriostasis and required for optimal neutrophil function. Prolactin, a lactogenic hormone, regulates the output of insulin-like growth factor-1. Genetic ablation of Prlr results in mice which show multiple defects in reproduction leading to infertility, altered maternal behavior and reduced bone development (Brooks 2012; Binart et al. 2010; Bole-Feysot et al. 1998). Of importance, Ltf and Prlr in the liver were decreased significantly in G3 HO but only Prlr was restored by taurine-supplementation (G3 HOT) (Fig. 4) at PD1. However, at 1 M, both Prlr and Ltf were decreased significantly compared to WT and restored to WT with added taurine, suggesting these genes may be down-regulated by low taurine concentrations. Upp 2 catalyzes the cleavage of uridine to uracil and ribose and used both energy sources and nucleotide synthesis (Roosild et al. 2011; Wan et al. 2010; Johansson 2003). Serine is a substrate for Sds with production of the two products, pyruvate (precursor of glucose) and NH_3 for gluconeogenesis from amino acids (Masuda et al. 2003; Imai et al. 2003; Lopez-Flores et al. 2006) The significant increase of Upp 2 and Sds in G3 HO without MS is unclear but may show the

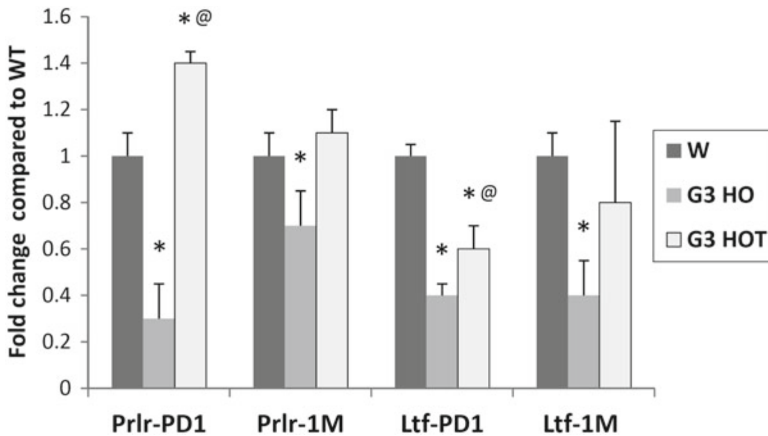

Fig. 4 Fold changes of Prlr and Ltf in G3 HO and G3 HOT compared to those in WT at PD1 and 1 M. The liver at PD 1 and 1 M were homogenized with Trizol and mRNA was extracted using RNeasy kit. After cDNA was produced by reverse transcriptase, 10 ng of cDNA was mixed with SYBR master mix and reacted with primers using PCR system. Data are expressed mean ± SE as fold change compared to WT. * is significantly ($p < 0.001$) different compared to WT, and @ is significantly ($p < 0.01$) different compared to G3 HO

critical regulation of physiological processes by uridine or may be due to starvation (Ishikawa et al. 1965). However, Upp2 and Sds in the liver from G3 HO at 1 M were not changed although expression of these two enzymes in the liver from G3 HO without MS at PD1 is dramatically increased. With taurine supplementation both genes were significantly decreased, suggesting taurine may be involved in regulation of nucleotide metabolism and gluconeogenesis.

Alteration of gene expression in CSAD KO was similar to that in other models of taurine deficiency. Gene expression in CDO KO was altered compared to WT. Expression of CSAD gene in CDO KO was increased significantly compared to WT and expression of ADO gene in CDO KO was without effect similar to CSAD KO. Although an increase of TauT in CDO KO was consistent with that in CSAD KO, TauT in taurine-treated CDO KO was restored to WT (Roman et al. 2013) but TauT in taurine-treated CSAD KO was not restored. This difference may be attributed to differences in the amount of taurine used; CSAD KO and CDO KO were treated with 0.05 and 2 % in the drinking water, respectively (Ueki et al. 2011).

5 Conclusion

Newborn G3 HO and G4 HO mice with a milk spot (MS) survive and thrive to grow well as we demonstrated previously. Surviving CSAD KO at 1 M after weaning indicated that taurine is redistributed on the basis of need, regardless of its origin, which suggests that the requirement for taurine for homeostasis and survival may

vary from organ to organ. Although CSAD KO has low reproductive performance, surviving CSAD KO mice are good models for understanding the physiological role of taurine in various disorders as well as for examining gene regulation in the absence of CSAD and/or low taurine levels. Supplementation of taurine in the food and drinking water may provide a potential prevention and treatment of various disorders caused by taurine deficiency, especially prior to or during pregnancy.

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A Novel Cysteine Sulfinic Acid Decarboxylase Knock-Out Mouse: Pathology of the Kidney and Lung in Newborn Pups

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Abbreviations

CDO	Cysteine dioxygenase
CDO KO	Cysteine dioxygenase knockout mice
CSAD	Cysteine sulfinic acid decarboxylase
CSAD KO	Cysteine sulfinic acid decarboxylase knockout mice
G1 G2, G3	Generation 1, 2 and 3
G1 HO	HO (CSAD$^{-/-}$) mice born from HT (CSAD$^{+/-}$) parents
G2 or G3 HO	Mice born from G1 HO or G2 HO parents
HO	Homozygotic mice (CSAD$^{-/-}$)
HOT	Homozygotic mice treated with 0.05 % taurine
HT	Heterozygotic mice (CSAD$^{+/-}$)
WT	Wild type (CSAD$^{+/+}$)

1 Introduction

Taurine, a sulfur containing amino acid restricted to the animal kingdom, is naturally produced by most mammals and it is not incorporated into proteins (Sturman 1993; Huxtable 2000). Taurine is produced in the liver from methionine and

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cysteine via the enzyme cysteine dioxygenase (CDO) and cysteinesulfinic acid decarboxylase (CSAD) (Schuller-Levis and Park 2003, 2006; Stipanuk 2004). Taurine plays essential roles in many physiological functions (Sturman 1993; Schuller-Levis and Park 2006). Recent efforts have been made to understand the various roles of taurine in biological processes using knockout mice (KO) including CDO KO and taurine transporter KO produced by gene targeting (Ueki et al. 2011; Heller-Stilb et al. 2002; Ito et al. 2008). Early studies of taurine used cats because the feline does not self-produce taurine (Sturman 1993). Taurine deficient cats demonstrated visual deterioration due to oxidant injury to the retina, suggesting taurine has antioxidant activities.

Our laboratory demonstrated that in the lungs taurine acts as an antioxidant by reducing inflammation and fibrosis. Thus, taurine effectively prevents lung injury due to oxidative damage from ozone, nitrogen dioxide, paraquat, amiodarone, and bleomycin when combined with niacin (Gordon et al. 1992, 1998; Wang et al. 1991; Schuller-Levis et al. 1995, 2003, 2009). In addition to its antioxidant properties, taurine is important in normal function in the kidney as it helps regulate osmoregulation (Chesney et al. 2010; Burg et al. 2007; Uchida et al. 1992; Burg 1994; Handler and Kwon 1997, 2001). Data show that taurine, a metabolically inert substance that is not incorporated into proteins and is not catabolised, a significant solute reserve for osmoregulation. The high concentrations of taurine in mammalian cells also supported this conclusion. Taurine has the potential to be a regulator of cell volume and an important amino acid in osmoregulation. One experiment involving renal tubular cell cultures demonstrated taurine increased cell viability during hypoxic preservation and after reperfusion reduced osmotic swelling and maintained intracellular calcium homeostasis (Michalk et al. 1996). Additional data show that the taurine positively affected organ preservation in isolated perfused kidneys, and reduced cellular injury induced by reperfusion and ischemia (Mozaffari et al. 2010). As an antioxidant in the kidney taurine prevents lipid peroxidation in glomerular mesangial cells and tubular epithelial cells that are exposed to an excess of glucose (Trachtman et al. 1993). Supplementation with taurine helps to ameliorate renal diseases such as nephrosis and diabetic nephropathy (Koh et al. 2008; Han and Chesney 2012; Choi et. al 2014).

If taurine plays essential roles involving antioxidation and osmoregulation within the kidneys and lungs, G3 HO of CSAD KO developed in our laboratory may demonstrate damage in both kidney and lung, compared to WT (Park et al. 2014a). Fetuses with access to utero taurine, G3 HOT from the dam, will undergo less degeneration of the kidney and lung.

2 Materials and Methods

2.1 Materials

Chemicals used in this study were purchased from Sigma Chemicals (St. Louise, MO) if not otherwise noted.

2.2 CSAD KO Mice

CSAD KO mice were produced from our laboratory as previously described (Park et al. 2014a). Briefly, chimeric CSAD KO mice were produced by injection of cells from a gene trap ES cell line (XP0392) into C57BL/6 (B6) blastocysts at the Mouse Mutant Regional Resource Centers (MMRRC, UC Davis, CA) which were implanted into a pseudopregnant B6 mouse. Chimeric mice from MMRRC were mated with B6 (Jackson Laboratories, Bar Harbor, ME) in the animal colony at NYS Institute for Basic Research in Developmental Disabilities. Heterozygous siblings were mated to produce $CSAD^{-/-}$ homozygous pups (HO). Experimental mice were fed taurine-free chow (LabDiet^{R}, PMI Nutrition International, St. Louis, MO). Taurine concentrations in commercial food were confirmed by HPLC. All mice were kept under 12-h day/night with free access to food and water. Some experimental animals had exogenous taurine added to their water at 0.05 % as indicated in Results. For optimum reproductive performance, one or two females were mated to a single male. Both females and males used for mating in the taurine-treated groups were supplemented with taurine. Newborn pups without a milk spot from G3 HO and newborn pups with a milk spot from WT and G3 HOT were used for pathology of the lung and kidney in order to examine the cause of death. All procedures involving live animals were approved by the Institutional Animal Care and Use Committee of IBR.

2.3 Light and Electron Microscopy

Light microscopy was performed using the lung and kidney of each mice at birth fixed with 10 % buffered formalin. The specimen for electron microscopy was fixed additionally with a mixture of 4 % paraformaldehyde and 1 % glutaraldehyde (Schuller-Levis et al. 2009). The tissues were then sliced with a double edged razor blade and put into 3 % glutaraldehyde in an 0.2 M sodium cacodylate buffer for overnight fixation. The tissues were further sliced no thicker than 2 mm and returned to the glutaraldehyde fixative for 3 h. The tissue was then treated with 1 % osmium tetroxide for 1 h except the brain which was treated with osmium for 2 h. The tissue was washed with cacodylate buffer and then treated with steps of ethanol, 50 %, 70 %, 95 % and 3 changes in 100 % for 10 min. The tissue was treated for 10 min in propylene oxide for 10 min and into 1:1 propylene oxide:epon for 1 h and into epon and polymerized overnight at 70 °C. One micrometer sections were cut, stained with methylene blue and Azure II and observed by light microscopy. Representative areas were chosen for ultrathin sectioning. The sections were stained with uranyl acetate and lead citrate and observed with an H-7000 Hitachi TEM. Histopathology was evaluated twice and independently by two investigators, who were blind to the data groups with an inter-observer agreement of 100 %.

3 Results and Discussion

3.1 *Morphological Abnormalities at Birth in the Kidney from G3 HO and G3 HOT*

The kidneys from G3 HO exhibit distal tubule changes (Fig. 1). The distal tubules show complete cell destruction and the lingering fragments of intracellular matter. Most of the changes are reflected in the form of necrosis or cell death of the distal tubule epithelium. G3 HOT show the same necrotic distal tubule alterations (Fig. 2). Normal kidney from WT shows normal uninjured distal tubule epithelium (Fig. 3a, b). The glomeruli and other tubules in the cortex remain unchanged in WT.

This effect on the distal tubule was an expected result considering that the functions of distal tubules include the reabsorption of nutrients and water from the urine are dependent upon the hydration of the animal (Hayslett et al. 1967; Dantzler and Silbernagl 1976). The accumulation of taurine by distal tubular cells has been hypothesized as the mechanism which regulates cell volume during osmotic stress (Uchida et al. 1992; Burg 1994). Without taurine the distal tubules are dramatically affected by osmotic changes. Further, taurine supplementation of the parents during gestation was not sufficient to protect the distal tubules from the lack of taurine. Since there is a minimal inflammatory response, necrosis may be a result of apoptosis.

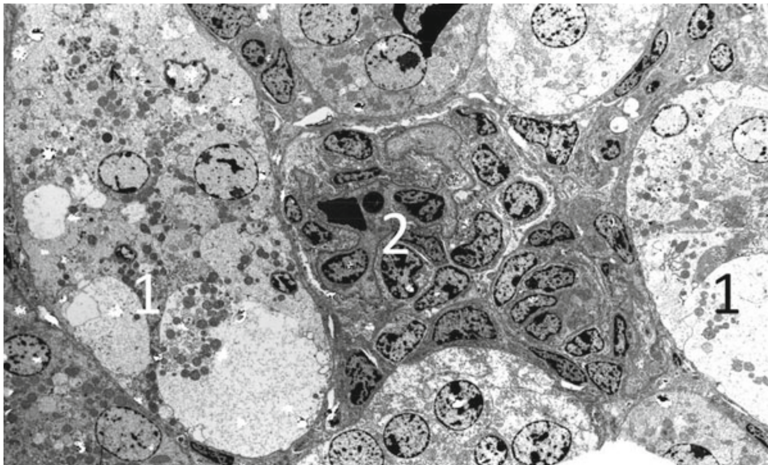

Fig. 1 Electron micrograph of distal tubules of the kidney in G3 HO. Distal tubules (*1*) show the complete cell destruction and the lingering fragments of intracellular matter in taurine deficiency due to lack of osmoregulation. Glomeruli (*2*) were intact. ×7,000

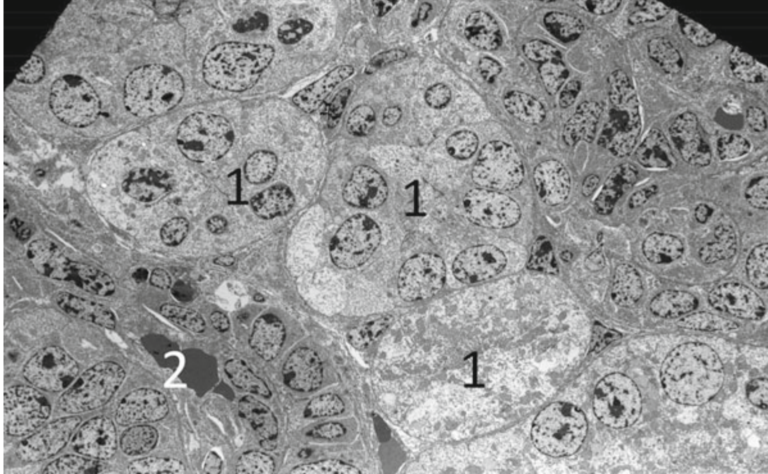

Fig. 2 Electron micrograph of distal tubules of the kidney in G3 HOT whose dam was treated with 0.05 % taurine in the drinking water. Distal tubules (*1*) show similar damage to G3 HO, Glomeruli (*2*) were intact. ×7,000

3.2 Morphological Injury at Birth in the Lung in G3 HO and G3 HOT

G3 HO (Figs. 4, 5, and 6) exhibit several abnormalities, supporting some of the potential biological roles of taurine. Figure 4 shows a neutrophil contacting a nearby endothelium cell as the initial process of neutrophil migration during inflammation similarly observed in bleomycin-induced inflammation (Schuller-Levis et al. 2009). In other sites there may be separation of the endothelium as the neutrophil migrates through the endothelium into the epithelium and the airspace. There was plasma leakage as evidenced by edema fluid (Figs. 5 and 6). The edema and the inflammation was a direct result of oxidant injury to both the epithelium and endothelium with the lack of antioxidant capacity or defective osmoregulation in the absence of taurine (Guler et al. 2014; Jeon et al. 2009; Yucel et al. 2008). There was little difference with respect to cell injury in G3 HO and G3 HOT (Figs. 7, 8, and 9), which demonstrate areas of edema. These data suggest 0.05 % taurine is not sufficient for protecting pulmonary injury in G3 HO. These abnormal accumulations of fluid may be the result of lung injury initiated on by the lack of antioxidation and/or insufficient osmoregulation. In addition, there was a noticeably greater amount of surfactant (Fig. 8) found in the experimental group compared to WT. The Clara cells have significantly more pooled cytoplasmic glycogen (Fig. 9) compared to WT (Figs. 10, 11, and 12). The images of WT showed intact and normal lung cells. In Fig. 10, the neutrophil does not come into contact with the surrounding endothelium cells but

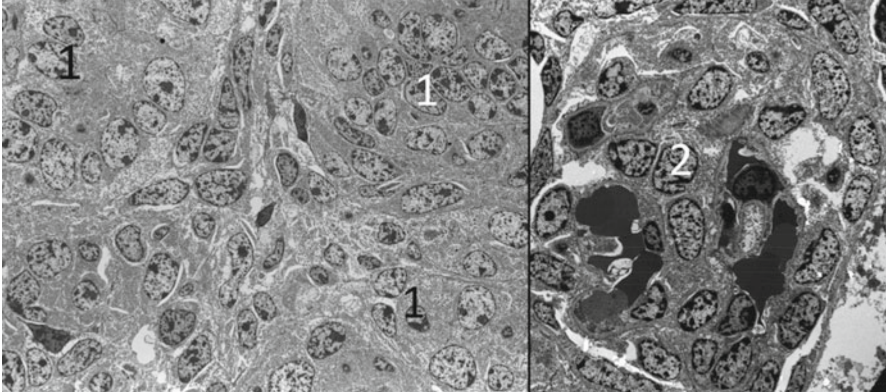

Fig. 3 Electron micrograph of distal tubules of the kidney in WT. Distal tubules (*1*) and glomeruli (*2*) were intact and unchanged in WT. ×7,000

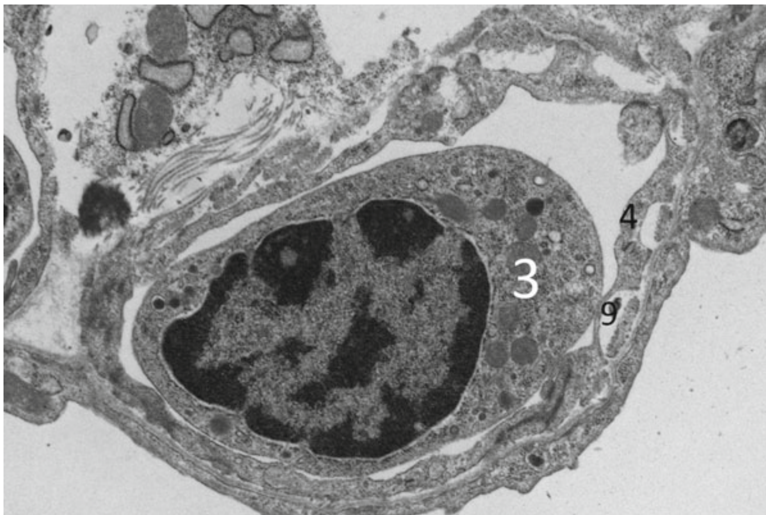

Fig. 4 Electron micrograph of the lung in G3 HO. A neutrophil (*3*) contacted a nearby endothelial cell (*4*) as evidence of oxidant injury, the initial process of neutrophil migration during inflammation. Edema (*9*) induced by plasma leakage was observed. ×8,000

fits well without contacting the endothelium, as expected in a normal lung. Figure 11 shows other areas of the lung that are also intact, including a capillary, surfactants, and Type II pneumocytes. It should be noted that there is no edema, or a swelling of an area caused by an excess of watery fluid (Fig. 11). The control (Fig. 12) shows normal Clara cells containing secretory granules, glycogen, and a nucleus.

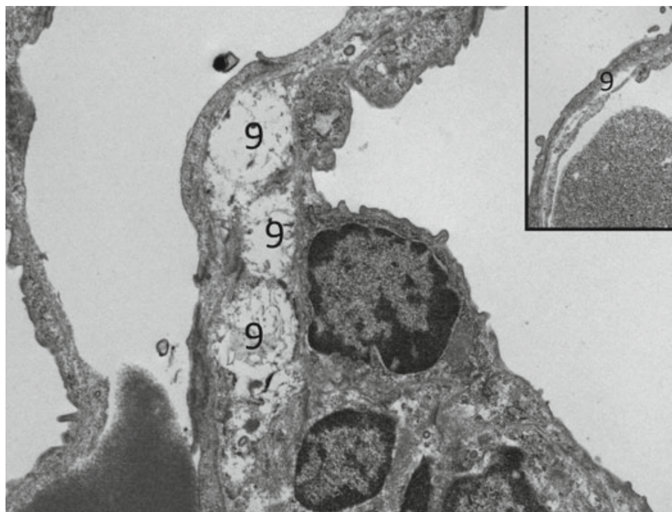

Fig. 5 Electron micrograph of the lung in G3 HO. Edema (*9*) was observed. ×6,000 and ×4,000 (*inlet*)

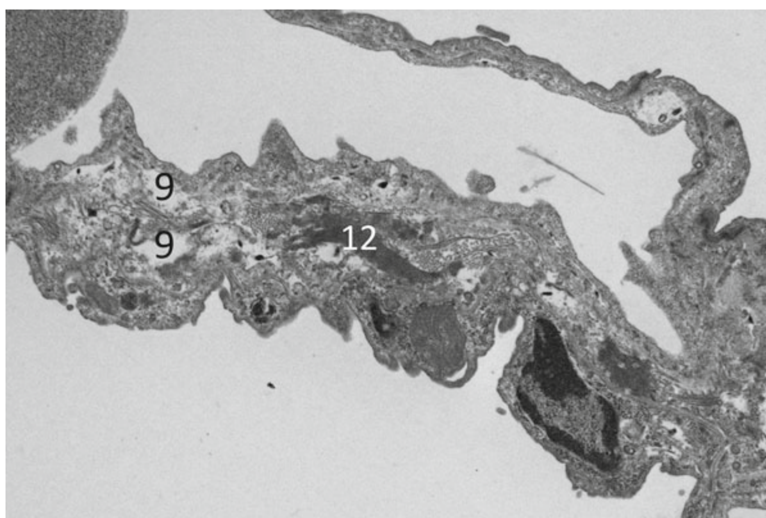

Fig. 6 Electron micrograph of the lung in G3 HO. Edema (*9*) and elastin (*12*) were observed. ×6,000

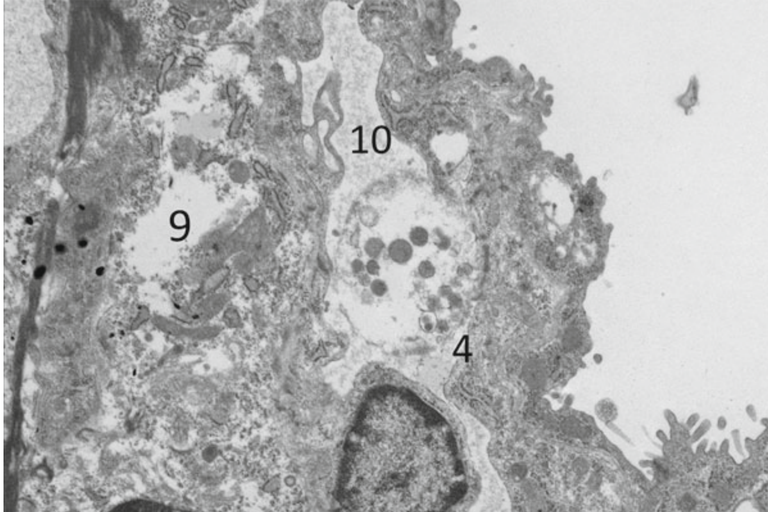

Fig. 7 Electron micrograph of the lung in G3 HOT whose dam was treated with taurine in the drinking water. Oxidant injury similar to G3 HO was observed. Edema (*9*), vascular space (*10*) and endothelium (*4*) were noted. ×6,000

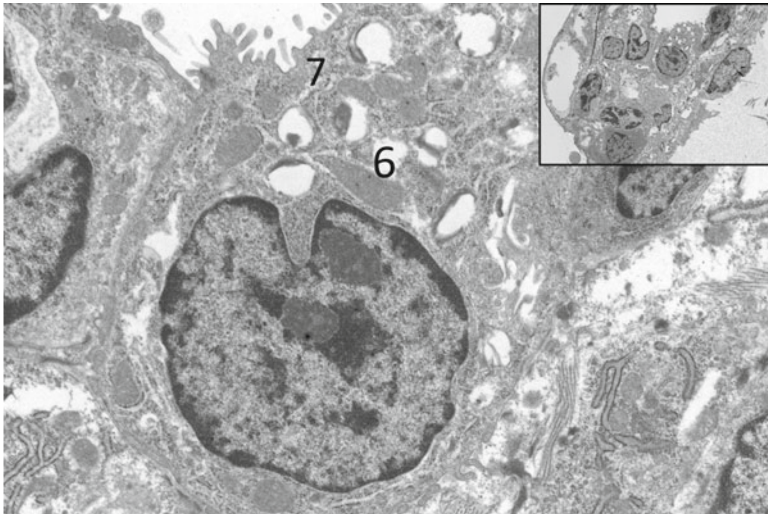

Fig. 8 Electron micrograph of the lung in G3 HOT. Type II pneumocytes (*7*) and surfactant (*6*) were shown. ×6,000 and ×2,000 (*inlet*)

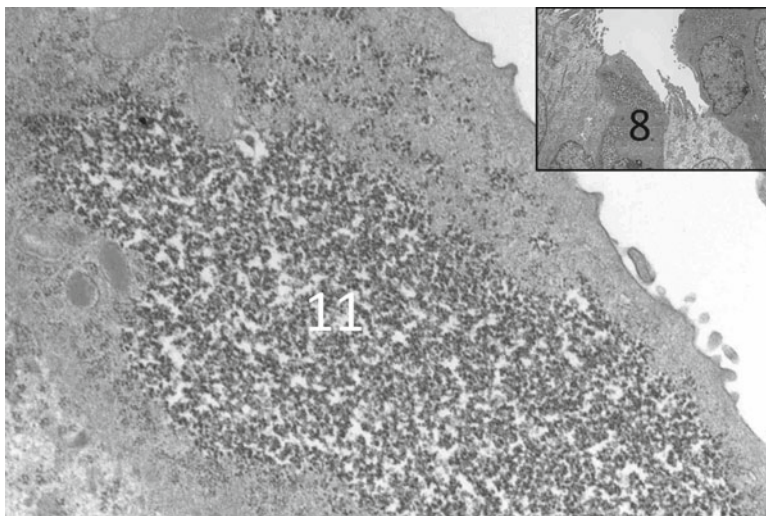

Fig. 9 Electron micrograph of the lung in G3 HOT. Glycogen accumulation (*11*) and Clara cell (*8*) were detected. ×12,000 and ×3,000 (*inlet*)

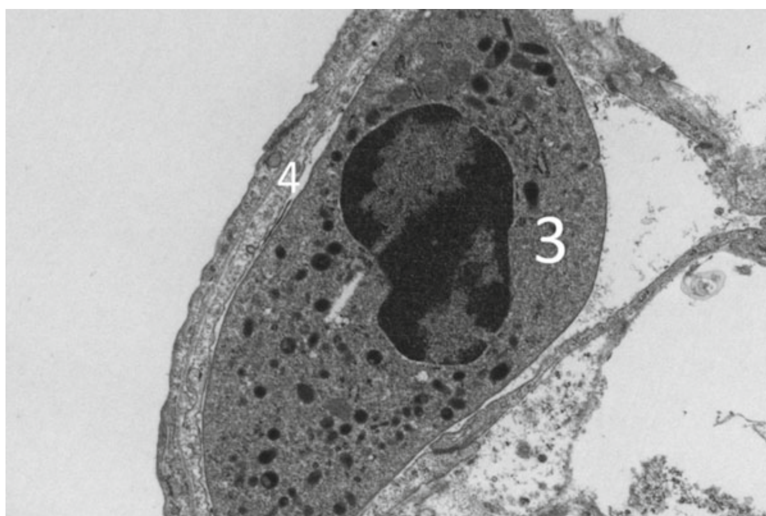

Fig. 10 Electron micrograph of the lung in WT. A neutrophil (*3*) does not come into contact with the surrounding endothelium cells, as expected in a normal lung. ×8,000

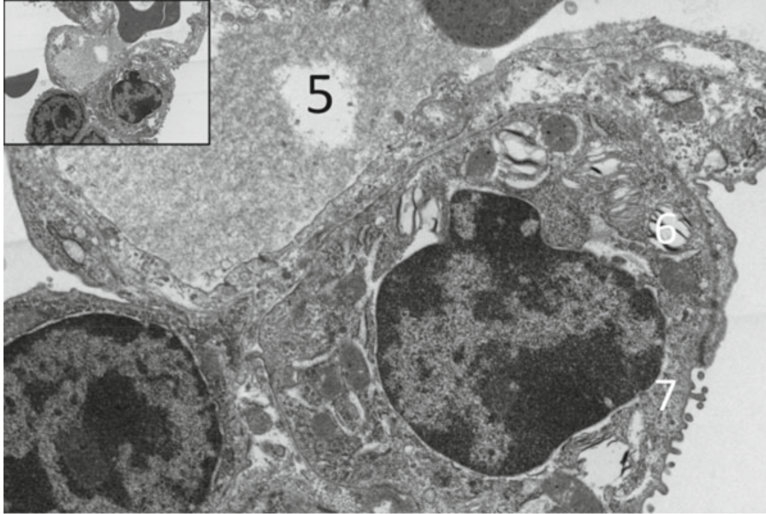

Fig. 11 Electron micrograph of the lung in WT. The lung in WT is intact, including a capillary (*5*), surfactants (*6*) and type II pneumocytes (*7*). ×6,000 and ×4,000 (*inlet*)

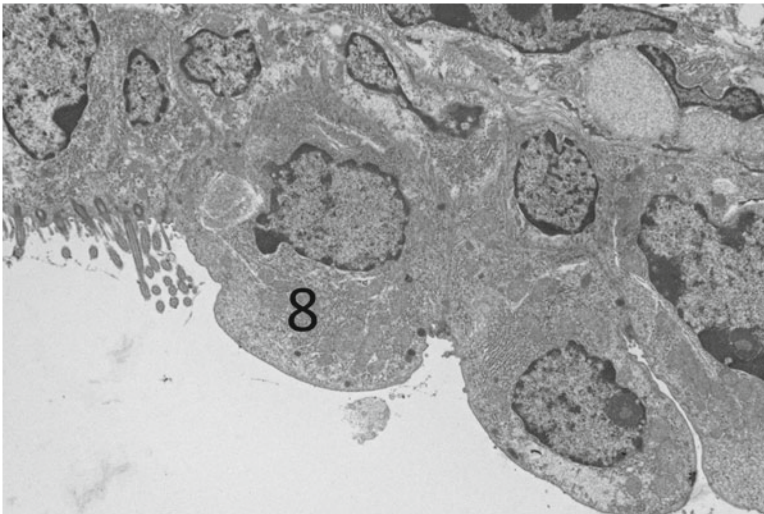

Fig. 12 Electron micrograph of the lung in WT. The Clara cells (*8*) are intact containing secretary granules, glycogen (*11*) and a nucleus. ×6,000

4 Conclusion

The lack of taurine has a substantial impact on both the kidney and lung with loss of function and eventual necrosis. The distal tubules of the kidney in taurine depleted mice showed cellular damage, indicating taurine has a critical role in the

osmoregulation of kidney distal tubules. In addition, taurine plays a critical role as an antioxidant in the lung. Both edema and contact of the neutrophil with the epithelial cells in G3 HO indicate oxidant injury and a lack of osmoregulation in the lung due to taurine deficiency. Since Clara cells are identified with homeostasis, it is possible that taurine deficiency resulted in an increase of glycogen as a mechanism of pulmonary host defense and epithelial repair.

This animal model parallels many diseases of the kidney and lung and will allow a better understanding of the role of taurine in various diseases as well as finding more effective ways of treatment and prevention.

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Insights into Taurine Synthesis and Function Based on Studies with Cysteine Dioxygenase (*CDO1*) Knockout Mice

Martha H. Stipanuk, Halina Jurkowska, Heather B. Roman, Julie Niewiadomski, and Lawrence L. Hirschberger

Abbreviations

ADO	2-Aminoethanethiol (cysteamine) dioxygenase
CDO	Cysteine dioxygenase
CSAD	Cysteinesulfinic acid decarboxylase

1 Introduction

In mammals, taurine can be synthesized by two pathways: (1) the conversion of cysteine to cysteinesulfinate by cysteine dioxygenase (CDO, EC 1.13.11.20, encoded by *Cdo1*), followed by its decarboxylation to hypotaurine by cysteinesulfinate decarboxylase (CSAD, EC 4.1.1.29, encoded by *Csad*) and the oxidation of hypotaurine to taurine, and (2) the incorporation of cysteine into coenzyme A, followed by the release of cysteamine during coenzyme A turnover, the oxidation of cysteamine to hypotaurine by cysteamine (2-aminoethanethiol) dioxygenase (ADO, EC 1.13.11.19, encoded by *Ado*), and the further oxidation of hypotaurine to taurine. ADO is expressed in a ubiquitious manner with high levels in most rat and mouse tissues, whereas CDO is expressed in a much more tissue- and cell-type-specific manner, particularly in liver, kidney, pancreas and adipose tissue (Sjoholm et al. 2006;

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Tsuboyama-Kasaoka et al. 2006; Ueki and Stipanuk 2007; Ide et al. 2002; Chen et al. 2000; Dominy et al. 2007; Coloso et al. 2006).

Over the past few decades, our understanding of cysteine metabolism and taurine synthesis has held that the liver is the major site of CDO expression, that the liver plays the major role in responding to changes in dietary sulfur amino acid levels by altering rates of cysteine catabolism by CDO, and that the CDO/CSAD pathway is the major route of taurine biosynthesis in vivo (Stipanuk et al. 2006; Tsuboyama-Kasaoka et al. 1999; Tsuboyama et al. 1996; Hirschberger et al. 2001). In isolated hepatocytes and in liver of intact rats, CDO concentration and activity undergo large fold changes in response to changes in cysteine availability. The regulation of CDO concentration occurs at the level of protein turnover via regulated ubiquitination and proteasomal degradation (Stipanuk et al. 2004; Dominy et al. 2006). Activity is further regulated via formation of a cysteinyl-tyrosine crosslink at the active site that dramatically increases CDO's catalytic efficiency (Dominy et al. 2008). Thus, CDO is rapidly degraded in hepatocytes cultured with a low cysteine concentration and in liver of animals fed a low protein diet, whereas CDO accumulates and is converted to its mature, more active form in hepatocytes cultured in medium with a higher cysteine concentration and in liver of animals fed a high protein diet. Hepatic CDO mRNA levels are largely unresponsive to changes in dietary protein or sulfur amino acid levels, consistent with its post-transcriptional/post-translational regulation (Lee et al. 2004; Bella et al. 1999a, b). Tissue ADO activity does not appear to respond to changes in dietary protein or sulfur amino acid level, allowing this minor pathway of taurine biosynthesis to function in conjunction with coenzyme A turnover (Coloso et al. 2006; Dominy et al. 2007).

We recently reported the development of *Cdo1* knockout mice as models for the study of disruptions of cysteine metabolism and taurine synthesis (Ueki et al. 2011, 2012). In this paper, we summarize some of our significant findings with regard to taurine metabolism that have resulted from the use of these mice.

2 Generation of *CDO1* Knockout Mice

Mouse chromosome 18 contains the murine *Cdo1* gene, which has five exons. Mice with a conditional *Cdo1* allele with loxP sequences flanking exon 3 of the murine *Cdo1* gene were generated and used to further generate germ-line and tissue-specific *Cdo1* knockouts (Ueki et al. 2011). Deletion of exon 3 leads to loss of active site resides and also shifts the reading frame for exons 4 and 5. Mice or tissues with a disrupted *Cdo1* gene do not express any detectable CDO protein.

The generation of germ-line *Cdo1* knockout mice has been described by Ueki et al. (2011). Heterozygous C57BL/6 mice carrying one wild-type *Cdo1* allele and one floxed *Cdo1* allele (*Cdo1+/Flox*) were bred to generate mice homozygous for the floxed *Cdo1* allele (*Cdo1Flox/Flox*). Then *Cdo1Flox/Flox* mice were crossed with *CMV-cre* (+) transgenic mice [B6.C-Tg6(CMV-cre)1 Cgn/J; Jackson Laboratory] to generate mice that positive for the Cre recombinase allele and that

contained a Cre-recombined (null) allele (*Cdo1+/−Cre*+). *Cdo1+/−Cre*+ mice were then bred to wild type mice, and offspring expressing a Cre-recombined (null) *Cdo1* allele and not carrying the Cre allele were selected (*Cdo1+/−*). The colony was expanded by breeding these *Cdo1+/−* mice, and the *Cdo1−/−* and *Cdo1+/+* mice used in our studies were generated by breeding *Cdo1+/−* mice. It should be noted that *Cdo1−/−* offspring have access to taurine synthesized by the dam during gestation and lactation and become taurine deficient only after being weaned to taurine-free diet.

The generation of hepatocyte-specific knockout mice has been described by Ueki et al. (2012). *Cdo1Flox/Flox* mice were crossed with C57BL/6-Tg(Alb-cre)21Mgn/J mice (Jackson Labs) that express a transgene for Cre recombinase driven by the albumin gene promoter (AlbCre). Offspring with the *Cdo1Flox/*+AlbCre+ genotype were further crossed to obtain *Cdo1Flox/Flox*AlbCre+ mice. Finally, *Cdo1Flox/Flox*AlbCre+ and *Cdo1Flox/Flox* mice were crossed to obtain the *Cdo1Flox/Flox* AlbCre+ and *Cdo1Flox/Flox* littermates used in our studies. Because albumin expression begins around the time of birth in mice, the disruption of the floxed CDO gene in hepatocytes occurs postnatally after Alb-promoter-driven expression of Cre recombinase begins.

Mice are normally maintained on a modified AIN93G diet (i.e., our basal diet) that contains 200 g vitamin free casein and 1.5 g L-cystine per kg diet; we routinely reduce the L-cystine to half of that in the AIN93G formulation in order to improve growth and survival of *Cdo1−/−* pups in our breeding colony. For the studies reported in this paper, mice were given a diet enriched in sulfur amino acids for 7 days prior to tissue collection for metabolite analyses. This enriched diet was the same as the basal diet except it was supplemented with additional L-methionine and L-cystine to increase the total sulfur amino acid level to 12.3 g methionine equivalents per kg diet [1 g of cyst(e)ine = 1.24 g methionine equivalents] compared to 8 g methionine equivalents per kg of the basal diet.

3 The Phenotype of *CDO1−/−* Mice

In this study we examined the effects of taurine supplementation on pancreatic remodeling. Mice were supplemented with taurine (0.05 %) in drinking water at the age of 4 weeks and remained on this diet for an additional for weeks. When mice were 2 months old, pancreas were removed and processed for histology. Pancreata were dissected attached to the pyloric region of the stomach and the duodenum. The initial part of the duodenum served to orient the pancreas for the sectional plane. The pancreas of mice is not encapsulated in connective tissue, and it was difficult to isolate the pancreas without the surrounding adipose tissue of the peritoneum. Each pancreas yielded approximately 150 sections. Quantification of the number of islets was based on determining the number of islets per section. Histological examination of pancreas from taurine-fed mice revealed a significant increase in their size (Fig. 1). The pancreas from these

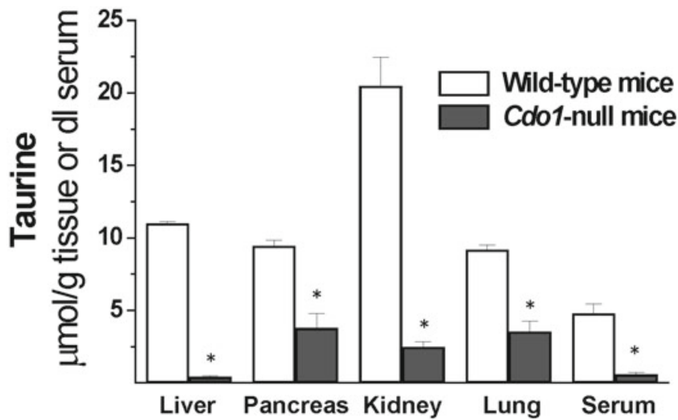

Fig. 1 Taurine levels in tissues from 8-week-old female *Cdo1*-null and wild-type mice. Values are means ± SEM for seven mice. *Values for *Cdo1*-null mice are significantly lower than those for wild-type mice ($p < 0.05$)

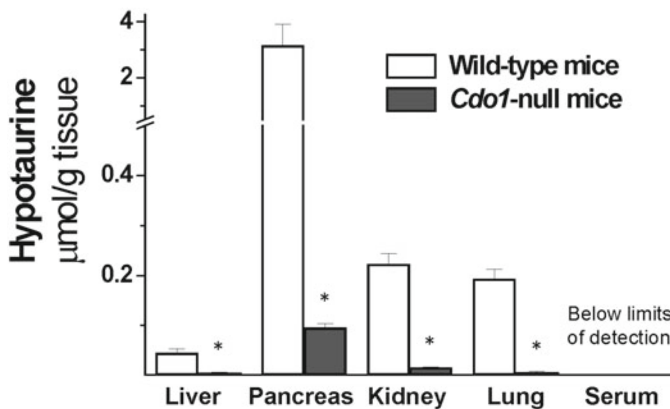

Fig. 2 Hypotaurine levels in tissues from 8-week-old female *Cdo1*-null and wild-type mice. Values are means ± SEM for seven mice. *Values for *Cdo1*-null mice are significantly lower than those for wild-type mice ($p < 0.05$)

mice did not seem to be enlarged and the exocrine serous acini were of normal histology when compared to the control pancreas. Interestingly however, the number of islets was significantly increased (Fig. 2). On average, a pancreas from control mice yielded approximately four islets per section, whereas a pancreas of taurine-fed mice contained more than ten islets. The number of islets per

section was not uniform throughout the pancreas, but differences in islets size and number between controls and taurine-fed mice was maintained proportionally throughout the different regions of the pancreas.

4 Mice Lacking CDO Have Low Taurine and Hypotaurine Levels

Taurine and hypotaurine in mouse tissues were measured by HPLC as described previously (Ueki et al. 2012). Taurine and hypotaurine levels in tissues from 8-week-old female mice are shown in Figs. 1 and 2 (based on data from Roman et al. 2013). Consistent with the lack of flux through the CDO-dependent pathway for taurine synthesis, *Cdo1*−/− mice fed a taurine-free diet have low taurine levels compared to *Cdo1*$^{+/+}$ mice, with liver values being less than 5 %, kidney and serum values being less than 15 %, and lung and pancreas values being less than 40 % of the respective control values. Hypotaurine levels were low, less than 7 % of wild-type levels regardless of the particular tissue, in *Cdo1*−/− mice. The proportionately greater drop in hypotaurine levels than in taurine levels in tissues of *Cdo1*−/− mice compared to wild-type controls, especially in pancreas and lung, reflects the fact that hypotaurine is an intermediate in biosynthesis of endogenous taurine. Taurine and hypotaurine levels in male *Cdo1*−/− mice were similarly low compared to male wild-type littermates (data not shown).

Unexpectedly, hypotaurine levels varied markedly among tissues. Those in the pancreas were 71- and 60-times those in liver for female and male mice, respectively, whereas kidney and lung had levels that ranged from 1.9- to 5.0-times the hepatic levels. In liver, kidney and lung of wild-type mice, the hypotaurine levels were less than 3 % of the taurine levels in the same tissue, whereas molar hypotaurine levels in the pancreas of wild-type mice were 33 % (female) and 86 % (male) of the pancreatic taurine levels. The identification of the hypotaurine peaks obtained by HPLC of samples from pancreas and other tissues was substantiated by conversion to taurine when samples were treated with H_2O_2 and is consistent with the dramatic reduction in size of the hypotaurine peak in tissues of *Cdo1*−/− mice.

Park et al. (2014) recently published their generation of a *Csad*−/− mouse which also has a block in taurine biosynthesis by the CDO/CSAD pathway. Liver taurine levels were much lower in 8-week old *Cdo1*−/− offspring of *Cdo1*+/− mice than they were in 8-week old *Csad*−/− offspring of *Csad*+/− mice (4 % compared to 74 % of wild-type control), while serum/plasma levels of taurine were similarly low compared to wild-type (12 % compared to 17 % of wild-type control). It is not at all clear why *Csad*−/− pups would be better able than would *Cdo1*−/− pups to conserve taurine obtained from its dam via the placenta or milk in order to maintain hepatic taurine levels. *Csad*−/− pups did have low brain taurine concentrations that were about 24 % of wild-type levels, a level of depletion that is in the range of what we observed for kidney, lung and pancreas of *Cdo1*−/− pups.

5 *CDO1−/−* Mice Have Altered Bile Acid and Organic Osmolyte Pools

Metabolite analyses were run on the LC-MS systems operated by the Broad Institute of Harvard and MIT. Male mice that were 10- to 11-weeks of age were euthanized with an overdose of isoflurane between 10:00 and 14:00 h, and tissues were rapidly removed and immediately frozen in liquid nitrogen and then stored at −80 °C until samples were analyzed. Metabolomic results were compared by Student's t-tests of log transformed values followed by correction for multiple testing/false discovery using the Benjamini and Hochberg correction procedure to determine metabolites with significant fold differences between *Cdo1−/−* and wild-type mice.

Metabolites that met the criterion for statistical significance and that also showed an average difference of at least twofold are listed in Table 1 (downregulated) and Table 2 (upregulated). As shown in Table 1, taurine was the mostly highly downregulated metabolite, with levels in *Cdo1*-null mouse liver being only 5 % of wild-type levels for liver and about 25 % for pancreas and epididymal fat. Putrescine (1,4-butanediamine), the product of ornithine decarboxylation, was also low in both liver and pancreas. The mostly highly upregulated metabolites in liver were unconjugated and glycine-conjugated bile acids, with unconjugated cholate/muricholate being 112-times as high in *Cdo1*-null mice as in wild-type mice and glycocholate/glycomuricholate being 30-times as high. For deoxycholate/chenodeoxycholate/ursodeoxycholate, the unconjugated forms were 6-times and the glycol-conjugated forms were 23-times as high. Taurine-conjugated bile acids were 60–70 % of wild-type levels and not significantly different between *Cdo1−/−* and *Cdo1+/+* mice.

Table 1 Downregulated metabolites[a] in tissues of male *Cdo1−/−* mice compared to male wild-type (*Cdo1+/+*) mice

Liver			Pancreas		
Metabolite	Ratio Null/WT	B-H corrected p value	Metabolite	Ratio Null/WT	B-H corrected p value
Taurine	0.05	6×10^{-9}	*Taurine*	0.23	0.000007
Isocitrate	0.26	0.034	*Putrescine*	0.45	0.002
1-Methyl-nicotinamide	0.40	0.035			
5-Hydroxy-tryptophan	0.40	0.009	Gonadal adipose tissue		
Putrescine	0.40	0.003	Metabolite	Ratio Null/WT	B-H corrected p value
Serotonin	0.42	0.010	*Taurine*	0.24	0.0005

[a]Metabolomic results (n = 5 for each genotype) were compared by Student's t-tests of log transformed values followed by correction for multiple testing/false discovery using the Benjamini and Hochberg correction procedure to determine metabolites with significant fold differences between *Cdo1−/−* and wild-type mice. Those metabolites that met the criterion for statistical significance and that also showed an average difference of at least twofold are listed in the table

Table 2 Upregulated metabolites[a] in tissues of male *Cdo1*−/− mice compared to male wild-type (*Cdo1*+/+) mice

Liver			Pancreas		
Metabolite	Ratio Null/WT	B-H corrected p value	Metabolite	Ratio Null/WT	B-H corrected p value
Alpha-glycerophospho-choline	2.24	0.009	Inositol	2.31	0.0003
Aminoisobutyric acid	2.25	0.0003	Beta-alanine	2.35	0.0005
Trimethyl-amine-N-oxide	2.50	0.037	*1-Methyl-nicotinamide*	2.76	0.0001
Orotate	2.59	0.025	*Trimethyl-amine-N-oxide*	2.90	0.003
Betaine	2.70	0.0007			
dTMP	2.79	0.013	Gonadal adipose tissue		
Lithocholate	3.01	0.0006	Metabolite	Ratio Null/WT	B-H corrected p value
2-Amino-adipate	4.21	0.042	Proline	2.02	0.04
Chenodeoxy-cholate/deoxycholate	6.09	0.0005	F1,6DP/F2,6D/G1,6DP	2.29	0.04
Glycodeoxy-cholate/ glycocheno-deoxycholate	23.15	0.004	*Quinolinate*	2.87	0.044
Glycocholate	30.32	0.027	Creatine	3.00	0.011
Cholate	112.19	0.00004	Carnosine	4.92	0.003
			Inositol	4.93	0.000005
			Trimethyl-amine-N-oxide	5.07	0.006
			Inositol	4.93	0.000005

[a]Metabolomic results (n = 5 for each genotype) were compared by Student's t-tests of log transformed values followed by correction for multiple testing/false discovery using the Benjamini and Hochberg correction procedure to determine metabolites with significant fold differences between *Cdo1*−/− and wild-type mice. Those metabolites that met the criterion for statistical significance and that also showed an average difference of at least twofold are listed in the table

The role of taurine in conjugation of bile acids is well-known. Although bile acid concentrations were not directly measured in our study, we estimated the distribution of the various bile acids and the concentrations in *Cdo1*-null mouse liver using the fold differences we observed along with the molar distributions reported by Alnouti et al. (2008) and Bobeldijk et al. (2008). Compared to their observations of ~98 % taurine-conjugated, <0.2 % glycine-conjugated and <3 % unconjugated bile acids in murine liver, we estimate that the hepatic bile acid pool in the male *Cdo1-null* mice was slightly higher at 1.5-times that in wild-type mice and comprised about 40 % taurine-conjugated, 4 % glycine-conjugated and 56 % unconjugated bile acids.

Present at concentrations in the range of 10–20 μmol per gram in murine tissues (Roman et al. 2013), taurine serves as a nonperturbing small organic molecule, or osmolyte. Compounds that might substitute for taurine as cellular organic osmolytes (Burg and Ferraris 2008) were also elevated in liver as well as pancreas and adipose tissue. Interestingly, trimethyl-amine-*N*-oxide was elevated in all three tissues, and inositol was elevated in both pancreas and adipose tissue. Trimethyl-amine-*N*-oxide is the oxidation product of trimethylamine. It is a common osmolyte in salt-water animals. In mammals, gut bacteria convert carnitine or choline to trimethylamine, and the trimethylamine is then oxidized to the *N*-oxide by flavin containing monooxygenase 3 (Yancey et al. 2002), which has been shown to be highly upregulated by bile acids by a pathway involving farnesoid X receptor (FXR) (Bennett et al. 2013). Inositol is an important osmolyte in mammalian tissues, and its tissue concentration is upregulated by an increase in the number of sodium myo-inositol transporters (SMITs) (Burg et al. 2007). Other upregulated osmolytes in tissues of *Cdo1−/−* mice were amino acids and amino acid metabolites, alpha-glycerophosphocholine, betaine, choline and creatine. The observed upregulation of a variety of compounds known to function as cellular osmolytes is consistent with the well-established role of taurine as a major organic intracellular osmolyte.

6 Hepatocyte-Specific Knockout of *CDO1* Results in Enhanced Levels of CDO in Nonhepatic Tissues

Despite the expression of CDO in several tissues, studies in intact rats fed different levels of dietary protein or sulfur amino acids indicated that only hepatic CDO changed in response to the changes in sulfur amino acid intake (Stipanuk et al. 2002). In rats fed a diet containing 400 g casein/kg, hepatic CDO activity was 34-times and CDO protein abundance was 12.5-times the levels observed in rats fed a diet containing 100 g casein/kg. These changes in CDO were associated with hepatic cysteine, glutathione and taurine levels that were 3.5-, 2.2- and 21.2-times, respectively, those in rats fed the low protein diet. In the same study, extrahepatic tissues (kidney, lung, brain) showed no changes in CDO abundance or activity and no changes in the tissue concentrations of cysteine, glutathione or taurine, except for a somewhat higher renal taurine level. The lack of response of extrahepatic

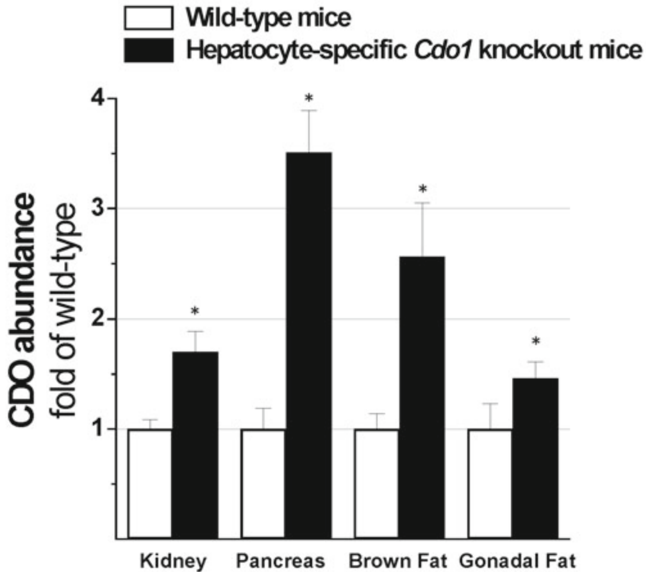

Fig. 3 CDO abundance in extrahepatic tissues of hepatocyte-specific *Cdo1* knockout mice. Values are means ± SEM for four to seven mice. *Values for hepatocyte-specific *Cdo1* knockout mice are significantly greater than those for wild-type mice ($p < 0.05$)

tissues may be a reflection of the efficiency of liver in removing sulfur amino acids from the portal circulation, converting them to glutathione, taurine and sulfate (Garcia and Stipanuk 1992).

Because other cells that express CDO, such as adipocytes, respond to changes in cysteine concentration in vitro (Ueki and Stipanuk 2007), we hypothesized that plasma cysteine levels would increase in mice without hepatic CDO and that this would then result in an increase in CDO abundance and activity in other tissues. Thus CDO expression was assessed in tissues of 8-week old mice that had been fed the diet enriched in sulfur amino acids for the previous 7 days. Mice were euthanized with CO_2, and tissues were collected and immediately frozen in liquid nitrogen.

The abundance of CDO protein and mRNA in kidney, pancreas, brown fat and gonadal fat were measured as described previously (Ueki et al. 2012). CDO mRNA was not different in the control and liver-specific *Cdo1* knockout mice, consistent with previous results demonstrating that the upregulation of CDO protein abundance by cysteine is not transcriptional. However, CDO protein was markedly upregulated in all four extrahepatic tissues that we tested, demonstrating that CDO can be upregulated in these tissues in the absence of hepatic CDO (Fig. 3). Plasma and liver cysteine levels were significantly higher in the liver knockout mice, but cysteine levels in kidney and pancreas were normal. Hypotaurine levels in kidney and pancreas were significantly higher in the liver *Cdo1* knockout mice, consistent with the increased abundance of CDO in these tissues and increased flux through the CDO-CSAD pathway.

7 Conclusion

The *Cdo1*−/− mouse, along with the *Csad*−/− mouse generated by Park et al. (2014) offer the opportunity to use genetic models to study the effects of blocks in taurine biosynthesis in vivo. These models provide approaches that complement that provided by the taurine transporter knockout mouse developed previously by Warskulat and coworkers (2007), which exhibits taurine deficiency mainly in tissues that cannot synthesize taurine and are thus largely dependent upon taurine uptake.

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Oxidation of Hypotaurine and Cysteine Sulfinic Acid by Peroxidase-generated Reactive Species

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Abbreviations

HRP	Horseradish peroxidase
HTAU	Hypotaurine
CSA	Cysteine sulfinic acid
TAU	Taurine
CA	Cysteic acid
Tyr	Tyrosine

1 Introduction

In the biochemical production of taurine (TAU), the oxidation of the sulfinic group of metabolic intermediates to the sulfonic group plays a crucial role both in the final step, where hypotaurine (HTAU) is oxidized to TAU, and in the intermediate step, where cysteine sulfinic acid (CSA) is oxidized to cysteic acid (CA) which is subsequently decarboxylated into TAU (Stipanuk and Ueki 2011). Although the oxidation of sulfinates (RSO_2^-) to sulfonates (RSO_3^-) has been well characterized, no specific enzyme has been detected so far for the sulfinate oxidation (Huxtable 1992). However, oxidizing agents, such as hydroxyl radical (Aruoma et al. 1988), photochemically generated singlet oxygen (Pecci et al. 1999) and peroxynitrite (Fontana et al. 2005) have been reported to accomplish such oxidation in good yield. In particular, the peroxynitrite-mediated oxidation of sulfinates may take

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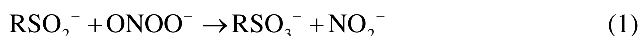
place either by two-electron mechanism (reaction 1) or by an indirect one-electron transfer mechanism involving hydroxyl (•OH) and nitrogen dioxide (•NO_2) radicals released during peroxynitrite homolysis or carbonate radical anion (CO_3•$^-$) generated by the short-lived peroxynitrite-CO_2 adduct (reaction 2) (Fontana et al. 2005; Fontana et al. 2006).

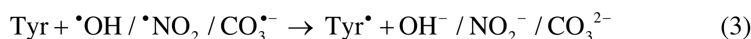


$$RSO_2^- + ONOO^- \rightarrow RSO_3^- + NO_2^- \quad (1)$$

$$RSO_2^- + {}^{\bullet}OH / {}^{\bullet}NO_2 / CO_3^{\bullet -} \rightarrow RSO_2^{\bullet} + OH^- / NO_2^- / CO_3^{2-} \quad (2)$$

We have also shown that HTAU and CSA are capable of preventing peroxynitrite-mediated oxidative/nitrative damage such as α_1-antiproteinase inactivation, human LDL oxidative modification, and tyrosine nitration (Fontana et al. 2004).

Peroxynitrite and its derived radicals, such as nitrogen dioxide radical (•NO_2), are well known as reactive species capable to initiate both oxidation and nitration reactions (Ducrocq et al. 1999). Protein tyrosine nitration was originally described in biology to occur via peroxynitrite-dependent reactions (Ischiropoulos et al. 1992; Goldstein et al. 2000; Reiter et al. 2000), but it can occur also due to other NO-mediated processes, most notably in heme peroxidase-catalyzed reactions (Wu et al. 1999; Brennan et al. 2002; Eiserich et al. 2002). The typical mechanism of tyrosine (Tyr) nitration in biological systems is a two-step radical process: one-electron oxidant leading to the formation of a tyrosyl radical (Tyr•) (reaction 3) which then combines at diffusion-controlled rates with •NO_2 to yield 3-nitrotyrosine (reaction 4).



$$Tyr + {}^{\bullet}OH / {}^{\bullet}NO_2 / CO_3^{\bullet -} \rightarrow Tyr^{\bullet} + OH^- / NO_2^- / CO_3^{2-} \quad (3)$$

$$Tyr^{\bullet} + {}^{\bullet}NO_2 \rightarrow 3-\text{nitrotyrosine} \quad (4)$$

The pathological role of •NO_2 has been mostly related to the increased level of nitrated tyrosine residues in proteins detected in many diseases (Beckmann et al. 1994). Nitrite (NO_2^-) is the major end product of NO metabolism and it can be detected in plasma, saliva, gastric juice and respiratory system lining fluid at μM levels (Green et al. 1982; Gaston et al. 1993; Leone et al. 1994; Ueda et al. 1995). Moreover, NO_2^- oxidation could be important at sites of inflammatory processes, when NO and nitrite levels are increased and myeloperoxidase is secreted from activated granulocytes (Farrell et al. 1992; Torre et al. 1996). Indeed, nitrite oxidation process can be catalyzed by heme peroxidases, horseradish peroxidase (HRP), myeloperoxidase, and lactoperoxidase in the presence of hydrogen peroxide (H_2O_2) (van der Vliet et al. 1997; Sampson et al. 1998; Gaut et al. 2002). It is well known that peroxidases generate nitrating and oxidizing species in vitro and in vivo, by a pathway involving nitrite (van der Vliet et al. 1997). The existence of two competing pathways has been reported, in which the nitrating and oxidizing species is either nitrogen dioxide or peroxynitrite. The first pathway involves one-electron

oxidation of nitrite by the classical peroxidase intermediates, compound I and compound II, whereas in the second pathway peroxynitrite is generated by reaction between enzyme-bound nitrite and hydrogen peroxide. The two mechanisms can be operative simultaneously, and their relative importance depends on the reagent concentrations. The peroxynitrite pathway takes place significantly only at relatively high nitrite concentrations (Casella et al. 2002).

The purpose of the present study was to investigate the oxidation of sulfinates, HTAU and CSA by peroxidase-generated reactive species. The results indicate that the formation of reactive nitrogen intermediates via peroxidase-catalyzed oxidation of nitrite could represents an additional mechanism leading to the formation of taurine. Moreover, the effect of sulfinates, HTAU and CSA, on nitrotyrosine formation by HRP/H_2O_2/nitrite system has been also studied. Noteworthy, the results provide evidence that sulfinates are able to inhibit nitrotyrosine formation not only by scavenging reactive nitrogen species but also by reducing the peroxidase-generated tyrosyl radical. Sulfinates are consequently oxidized to the respective sulfonates by the interaction with peroxidase-generated reactive species.

2 Material and Methods

2.1 Chemicals

Hypotaurine, cysteine sulfinic acid, horseradish peroxidase (HRP) (EC 1.11.1.7), catalase, and diethylenetriaminepenta-acetic acid (DTPA) were obtained from Sigma (St. Louis, MO). L-tyrosine, 3-nitro-L-tyrosine, potassium nitrite and hydrogen peroxide (30 %) were purchased from Fluka (Buchs, Switzerland). H_2O_2 concentration was verified using UV absorption at 240 nm ($\varepsilon = 43.6\ M^{-1}\ cm^{-1}$) (Hildebrandt and Roots 1975). All other chemicals were of the highest purity commercially available. To avoid metal-catalyzed oxidative reactions, all samples contained 0.1 mM DTPA.

2.2 Oxidation of HTAU or CSA by HRP in the Presence of Nitrite

HTAU or CSA (at different concentrations) were incubated at 37 °C with HRP (44 μg/mL), potassium nitrite (10 mM) and H_2O_2 (1 mM) in 0.1 M K-phosphate buffer, pH 7.4, containing 0.1 mM DTPA. H_2O_2 addition was used to start the reaction. Aliquots of the reaction mixture were taken at 5 min incubation time and the reaction was stopped by dilution (1:10) with K-phosphate buffer (0.1 M, pH 7.4) containing 200 units/mL catalase. The samples were subsequently used for HPLC analysis.

2.3 Tyrosine Nitration by HRP and Nitrite

The reaction mixture contained tyrosine (1 mM), HRP (44 µg/mL), H_2O_2 (1 mM), and potassium nitrite (1 mM) in the absence (control) or in the presence of HTAU or CSA at various concentrations, in K-phosphate buffer (0.1 M, pH 7.4) containing 0.1 mM DTPA. After 5 min incubation at 37 °C, nitrotyrosine formation was quantified by HPLC.

2.4 Oxygen Uptake

Oxygen uptake was examined using a Gilson 5/6 oxygraph and measured with Clark-type oxygen electrode fitted to a water-jacketed sample cell (1.8 mL) at 37 °C. The saturation oxygen concentration at this temperature was taken as 235 µM.

2.5 HPLC Analyses

Analyses were performed with a Waters Chromatograph equipped with a model 600 pump, a model 600 gradient controller and a Waters 474 scanning fluorescence detector. TAU and CA were analyzed using *o*-phthaldialdehyde precolumn derivatization, as previously described (Hirschberger et al. 1985). Detection was performed at 340 nm (excitation) and 450 nm (emission). The column was a Simmetry C18 (4.6 mm × 250 mm), 5 µm (Waters). The mobile phases were: (A) 0.05 M sodium acetate (pH 5.5)/methanol (80:20, v/v), and (B) 0.05 M sodium acetate (pH 5.5)/methanol (20:80, v/v). The elution gradient was as follows: linear from A to 50 % B in 5 min followed by isocratic mode at 50 % B. Flow rate: 1 mL/min at room temperature (20 °C). The elution times of CA and TAU were 7.5 min, and 24.5 min, respectively.

Nitrotyrosine was analysed by using a Water Chromatograph equipped with a model 600 pump, and a model 600 gradient controller. The column was a Nova-pak C18 (3.9 mm × 150 mm), 4 µM (Water). The mobile phase was: A, 50 mM K-phosphate/H_3PO_4, pH 3.0; B, acetonitrile:water (50:50, v/v). A linear gradient from A to 33 % B for 10 min was used at a flow rate of 1 mL/min. Eluates were monitored at 274 and 360 nm using a Waters 996 photodiode array. Nitrotyrosine was quantified at 360 nm using Millenium 32 software. The elution time of nitrotyrosine was 9 min and concentrations were calculated from a standard curve.

2.6 Statistics

Results are expressed as means ± SEM for at least three separate experiments performed in duplicates. Graphics and data analysis were performed using GraphPad Prism 4 software.

3 Results

3.1 *Oxidation of Sulfinates by HRP/H_2O_2/Nitrite System*

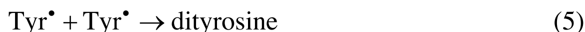
It is well known that peroxidases generate nitrating oxidants by a pathway involving nitrite (van der Vliet et al. 1997). The oxidation of the sulfinates, HTAU and CSA, by HRP/H_2O_2/nitrite system has been evaluated by monitoring the oxygen consumption and the production of the corresponding sulfonates, TAU and CA.

In the presence of H_2O_2, sulfinates are poorly oxidized by peroxidases (Fontana et al. 2008). However, when HTAU is reacted in a HRP/H_2O_2 system in the presence of nitrite, the production of TAU is observed after HPLC analysis of the reaction mixture. Figure 1 shows the amount of TAU produced after 5 min incubation in a enzymatic system containing HRP/H_2O_2 and 10 mM nitrite at pH 7.4. At 1 mM HTAU concentrations, the production of TAU was 138 ± 3 μM in an oxidative yield close to the 14 %. The same percentage value is found for the experiment carried out with 5 or 10 mM HTAU.

In Table 1, the oxygen uptake during the oxidation of HTAU by the HRP/H_2O_2/nitrite system is reported. The data show that TAU production is up to threefold higher that expected from the amount of oxygen consumed. On the contrary, CSA is poorly oxidized under the same experimental conditions (Fig. 1).

3.2 *Oxidation of Sulfinates by HRP/H_2O_2/Nitrite System: Effect of Tyrosine*

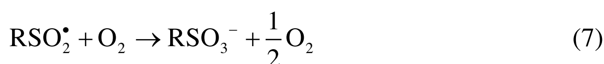
Oxidation of tyrosine by HRP in the presence of hydrogen peroxide forms tyrosyl radical, which dimerizes to form dityrosine. The dimerization reaction is not associated with oxygen consumption (reaction 5).



$$\mathrm{Tyr}^{\bullet} + \mathrm{Tyr}^{\bullet} \rightarrow \text{dityrosine} \tag{5}$$

However, it has been reported that, in the presence of sulfinates, HTAU or CSA, oxygen was consumed during the HRP/H_2O_2-dependent oxidation of tyrosine, indicating that the sulfinates have the ability to react with tyrosyl radical generated by peroxidase-catalyzed reaction (reaction 6). After this reaction, the formed sulfonyl radical (RSO_2•) reacts with oxygen, producing consequently the corresponding sulfonate (reaction 7) (Fontana et al. 2008).

$$\mathrm{RSO_2}^{-} + \mathrm{Tyr}^{\bullet} \rightarrow \mathrm{RSO}_2^{\bullet} + \mathrm{Tyr} \tag{6}$$



$$\mathrm{RSO}_2^{\bullet} + \mathrm{O_2} \rightarrow \mathrm{RSO_3}^{-} + \frac{1}{2}\mathrm{O_2} \tag{7}$$

Here, the oxidation of HTAU and CSA by the HRP/H_2O_2/nitrite system in the presence of tyrosine has been evaluated by measuring sulfonate production and oxygen

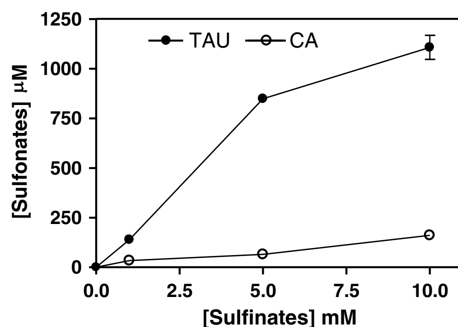


Fig. 1 Oxidation of sulfinates by HRP/H_2O_2 system in the presence of nitrite. HTAU or CSA, at different concentrations, were incubated with HRP (44 µg/mL) and H_2O_2 (1 mM) in the presence of K-nitrite (10 mM), in K-phosphate buffer (0.1 M, pH 7.4) containing DTPA (0.1 mM). After 5 min at 37 °C, TAU and CA production was determined by HPLC

Table 1 Oxygen uptake by HRP/H_2O_2/nitrite system

HTAU (mM)	O_2 uptake (µM)
1	52 ± 4
5	124 ± 11
10	156 ± 14

HTAU, at the indicated concentrations, was incubated in K-phosphate buffer (0.1 mM, pH 7.4) containing DTPA (0.1 mM) with HRP (44 µg/mL), K-nitrite (10 mM) and H_2O_2 (1 mM). The reactions were started by addition of H_2O_2 to the oxygraph chamber at 37 °C and the O_2 consumption was recorded for 5 min

consumption. Compared with the results obtained in the absence of tyrosine, the yield of TAU by the HRP/H_2O_2/nitrite system in the presence of 1 mM tyrosine is diminished (Fig. 2a) from 14 to 9.5 %. On the contrary, the amount of formed CA from CSA under the same experimental conditions is increased 1.6-fold (Fig. 2b). Interestingly, a high amount of oxygen consumption occurred during the cysteine sulfinic acid oxidation (Table 2).

3.3 Effect of Sulfinates on Tyrosine Nitration by HRP/H_2O_2/Nitrite System

In a previous paper, we reported that HTAU and CSA inhibited tyrosine nitration induced by peroxynitrite and peroxynitrite-CO_2 adduct (Fontana et al. 2004). However, the effect of sulfinates on tyrosine nitration by action of HRP/H_2O_2/nitrite

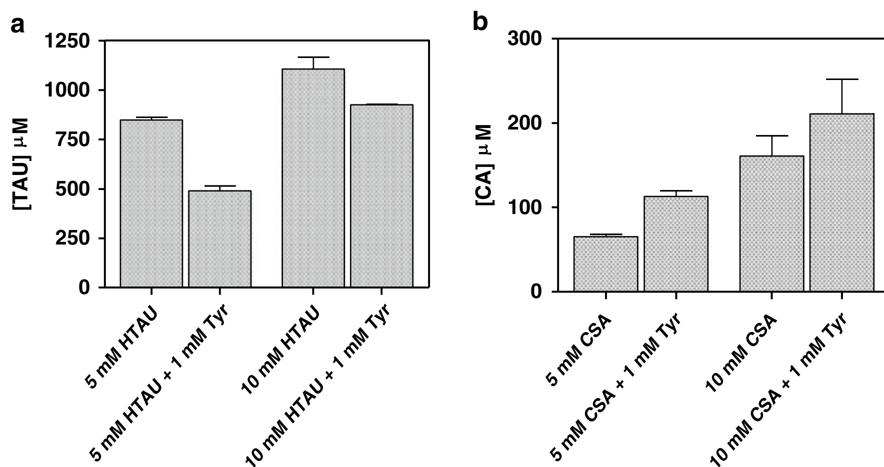


Fig. 2 Effect of tyrosine on the oxidation of sulfinates by HRP/H_2O_2/nitrite system. HTAU (**a**) or CSA (**b**), at the indicated concentrations, were incubated with HRP (44 μg/mL), K-nitrite (10 mM) and H_2O_2 (1 mM) in the presence or absence of Tyr (1 mM), in K-phosphate buffer (0.1 M, pH7.4) containing DTPA (0.1 mM). After 5 min at 37 °C, TAU and CA formation was quantified by HPLC

Table 2 Oxygen uptake by HRP/H_2O_2/nitrite system: effect of tyrosine

	O_2 uptake (μM)	
	Absence of Tyr	Presence of Tyr
CSA 10 mM	28 ± 3	236 ± 10
HTAU 10 mM	156 ± 14	113 ± 1

Sulfinates (10 mM), HTAU or CSA, were incubated in K-phosphate buffer (0.1 mM, pH 7.4) containing DTPA (0.1 mM) with HRP (44 μg/mL), K-nitrite (10 mM) and H_2O_2 (1 mM) in the presence or absence of tyrosine (1 mM). The reaction was started by addition of H_2O_2 to the oxygraph chamber at 37 °C and the O_2 consumption was recorded for 5 min

system has not been explored previously. To determine whether HTAU and CSA can compete with tyrosine in its ability to react with nitrating and oxidant species generated by HRP/H_2O_2 system, the effect of the two sulfinates on 3-nitrotyrosine formation has been investigated. Exposure of tyrosine (1 mM) to HRP/H_2O_2/nitrite resulted in the production of 5 ± 0.1 μM 3-nitrotyrosine, that was dose-dependently inhibited by HTAU or CSA added to the reaction mixture (Fig. 3). It could be observed that the ability of CSA to inhibit the formation of 3-nitrotyosine by HRP/H2O2/nitrite system was higher than that of HTAU.

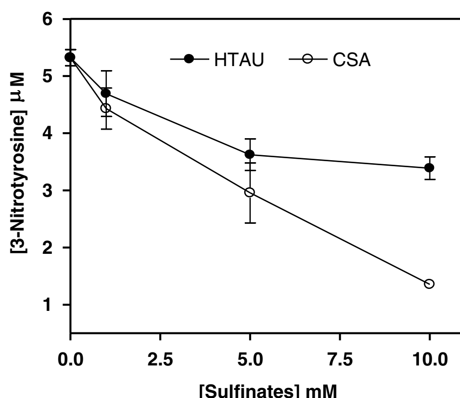
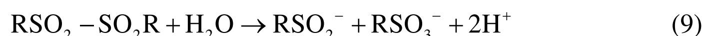


Fig. 3 Inhibition of tyrosine nitration by HRP/H_2O_2/nitrite system: effect of sulfinates. Tyrosine (1 mM) was incubated with HRP (44 μg/mL), H_2O_2 (1 mM), and K-nitrite (10 mM) in the absence or in the presence of HTAU or CSA at different concentrations, in K-phosphate buffer (0.1 M, pH 7.4) containing DTPA (0.1 mM). After 5 min at 37 °C, 3-nitrotyrosine formation was quantified by HPLC

4 Discussion

The results presented in this paper show that HTAU and CSA are oxidized to the respective sulfonates, TAU and CA, by peroxidase-generated reactive species. Previous works have shown that when sulfinates are oxidized by reactive nitrogen species such as peroxynitrite, a fast oxygen consumption is observed, suggesting that the peroxynitrite-mediated oxidation of sulfinates generates intermediate free radicals that react with oxygen (Fontana et al. 2005). Accordingly, the sulfinates can be indirectly oxidized to sulfonyl radicals by the peroxynitrite-derived free radicals, such as nitrogen dioxide and/or hydroxyl radicals (reaction 2). Consequently, sulfonyl radicals react with oxygen producing the corresponding sulfonates (reaction 7) (Fontana et al. 2005; Fontana et al. 2006). Other possible fate of sulfonyl radical is its dimerization to form the corresponding disulfone (RSO_2–SO_2R) that subsequently hydrolyzes to yield sulfonate (Fellman et al. 1987) (reaction 8 and 9).

$$RSO_2^{\bullet} + RSO_2^{\bullet} \rightarrow RSO_2 - SO_2R \tag{8}$$



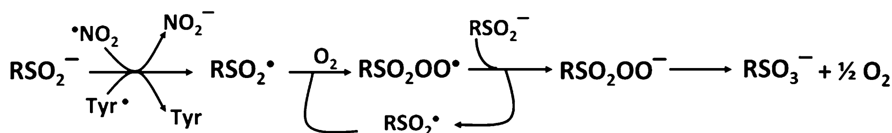
$$RSO_2 - SO_2R + H_2O \rightarrow RSO_2^{-} + RSO_3^{-} + 2H^{+} \tag{9}$$

This additional route does not require oxygen explaining the low oxygen consumption observed in our experiments (Table 1). Moreover, sulfinates can be oxidized directly (two-electron mechanism) to sulfonates by peroxynitrite, and in this case no oxygen uptake occurs (reaction 1). A second-order rate constant of 77.4 $M^{-1}s^{-1}$ and 76.4 $M^{-1}s^{-1}$ for HTAU and CSA, respectively, has been measured for this reaction

(Fontana et al. 2005). It has been already reported that HTAU and CSA are poorly oxidized by peroxidases and H_2O_2 (Fontana et al. 2008). Interestingly, we show that HTAU oxidation occurs at a good yield by the action of HRP/H_2O_2 system in the presence of nitrite (Fig. 1). During the oxidation of HTAU by the HRP/H_2O_2/nitrite system oxygen uptake has been observed suggesting the formation of transient radicals. Accordingly, nitrogen dioxide generated by peroxidase in this system can contribute to the oxidation of HTAU to TAU. Conversely, CSA is poorly oxidized under the same experimental conditions. This result is in accordance with the previous observation that nitrogen dioxide is a weak oxidant for this sulfinate (Fontana et al. 2008; Baseggio Conrado et al. 2014). At the same time, the lack of oxidation of CSA rules out a contribution of peroxynitrite pathway generated by our HRP/H_2O_2/nitrite experimental system. As the bimolecular constant of reaction of peroxynitrite with both sulfinates is similar, either CSA and HTAU should have been oxidized directly to the same extent.

Besides to reactive nitrogen species such as nitrogen dioxide, tyrosyl radicals are produced as a result of peroxidase-catalyzed tyrosine oxidation. It has been observed previously that the tyrosyl radicals generated by peroxidase/H_2O_2 system are reduced by sulfinates with generation of sulfonyl radical (RSO_2•) reacting rapidly with oxygen (reactions 6 and 7) (Fontana et al. 2008). Accordingly, we report the oxidation of both HTAU and CSA to the respective sulfonates, TAU and CA, by the HRP/H_2O_2/nitrite system in the presence of tyrosine. Noteworthy, when tyrosine is present, CSA is extensively oxidized as measured by CA production and oxygen consumption. This result suggests that CSA oxidation occurs via tyrosyl radical generated by peroxidase/H_2O_2/nitrite system (reactions 6 and 7), being poorly oxidized by peroxidase-generated reactive nitrogen species as reported above. HTAU oxidation is apparently not influenced by tyrosine presence under these experimental conditions. Indeed, in contrast to CSA, HTAU and tyrosine compete with nitrating oxidants generated by HRP/H_2O_2 system.

The capacity of sulfinates to protect against peroxynitrite-mediated tyrosine nitration has been also reported. The inhibition of tyrosine nitration exerted by HTAU and CSA was attributed mainly to their ability to scavenge hydroxyl and nitrogen dioxide radicals generated by peroxynitrite homolysis (Fontana et al. 2004). We show here, that HTAU and CSA are able to prevent tyrosine nitration induced by peroxidase-catalyzed oxidation of nitrite. According to oxygen uptake measurements, HTAU and CSA are able to reduce peroxidase-generated tyrosyl radicals (reaction 6). Consequently, the ability of sulfinates to react with tyrosyl radicals contributes to the protective effect of HTAU and CSA against tyrosine nitration. Therefore, sulfinates inhibit nitrotyrosine formation not only by scavenging reactive nitrogen species but also by reducing tyrosyl radicals. In addition, CSA inhibits tyrosine nitration more efficiently than HTAU. This finding can be explained by the different fate of CSA-derived sulfonyl radical that undergoes decomposition to yield sulfite as secondary product (Harman et al. 1984; Pecci et al. 1999). It has been reported that sulfite is an efficient protective agent against tyrosine oxidation induced by peroxynitrite-derived radicals (Fontana et al. 2008).



Scheme 1 Proposed pathway of sulfinate oxidation by peroxidase-generated reactive species

In conclusion, we propose that nitrogen dioxide (•NO_2) and/or tyrosyl (Tyr•) radicals oxidize by one-electron transfer mechanism the sulfinic group of sulfinates (RSO_2^-) to form a transient sulfonyl radical (RSO_2•) that initiates an oxygen-dependent radical chain reaction with sulfonates (RSO_3^-) as final products (Scheme 1). Furthermore, HTAU and CSA act as protective agents against peroxidase-generated radical species as evidenced by inhibition of tyrosine nitration.

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Increased *N*-Acetyltaurine in Serum and Urine After Endurance Exercise in Human

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Abbreviations

NAT	*N*-acetyltaurine
ACS2	Acetyl-CoA synthase 2
GM	Growth medium
DM	Differentiation medium

1 Introduction

Taurine (2-aminoethanesulfonic acid), which is the most abundant free amino acid-like compound found in mammalian tissues including liver and skeletal muscle (Awapara 1956; Jacobsen and Smith 1968; Huxtable 1980), has been previously reported to have many physiological and pharmacological actions (Pasantes et al. 1998; Huxtable 1992; Miyazaki and Matsuzaki 2014; Nakamura et al. 1993; Miyazaki 2010; Nieminen et al. 1988). The most established role of taurine is the conjugation with hydrophobic bile acids in the liver to increase hydrophilicity and facilitate excretion into the bile (Danielsson 1963; Sjovall 1959).

Recently, Shi et al. has reported that a novel metabolite *N*-acetyltaurine (NAT) that is *N*-acetylated form of taurine with acetate was increased in urine during alcohol catabolism (Shi et al. 2012). NAT is a highly hydrophilic and hygroscopic compound that has also found as a major compound in the viscid droplet of orb spider

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web (Vollrath et al. 1990; Higgins et al. 2001). The synthesis of NAT is depending on acetate level in the body, and acetate is produced from some metabolic sources including alcohol consumption (Buckley and Williamson 1977). Consumed alcohol is degraded to acetate via two-steps enzymatic metabolism in the liver. In addition, acetate is synthesized from acetyl-CoA by acetyl-CoA hydrolase in the liver and utilized as an alternative energy source via conversion to acetyl-CoA in the mitochondria of peripheral tissues by acetyl-CoA synthetase 2 (ACS2) under ketogenic conditions such as fasting and diabetes mellitus (Fukao et al. 2004; Luong et al. 2000; Sakakibara et al. 2009). Because the ketogenic condition would be induced during endurance exercise for continuous energy production in the skeletal muscle and heart, it is suggested that acetate is likely to be synthesized during endurance exercise. Therefore, there is a possibility that NAT biosynthesis might increase in result of *N*-acetylation of taurine with the elevated acetate or acetyl-group of AcCoA during the exercise in the skeletal muscles that abundantly contain taurine.

The present study purposed to determine the NAT in serum and urine after endurance exercises in humans, and in addition, to confirm the cellular production of NAT in skeletal muscle cell line following exposure to taurine and acetate, using HPLC-MS/MS analysis.

2 Methods

2.1 Serum NAT and Taurine Levels After Full-Marathon

Thirty-one healthy non-professional runners who participated in the 32nd Tsukuba Full-Marathon held in November 2012 were recruited as volunteers. Blood was collected from the participants at a day before, immediately after, and a day after the full-marathon race. Taurine and NAT in serum were measured by HPLC-MS/MS system. The study was carried out in accordance with the Declaration of Helsinki and was approved by the Human Subjects Committee of the University of Tsukuba. All subjects provided informed written consent.

2.2 NAT and Taurine Excretions in Urine After an Endurance Exercise

A healthy male (40 years of age) was loaded to a transient running. Experimental protocol was shown in Fig. 1. Before the exercise, urine was collected for 24 h between am11 on previous day and am11 on exercise day. At am11, blood was collected immediately before the exercise. Thereafter, the subject was loaded to run for an hour (running speed; approximately 9 km/h). Blood was also collected at immediately, 1, 2, 3, 4, and 24 h after the exercise. In addition, urine was

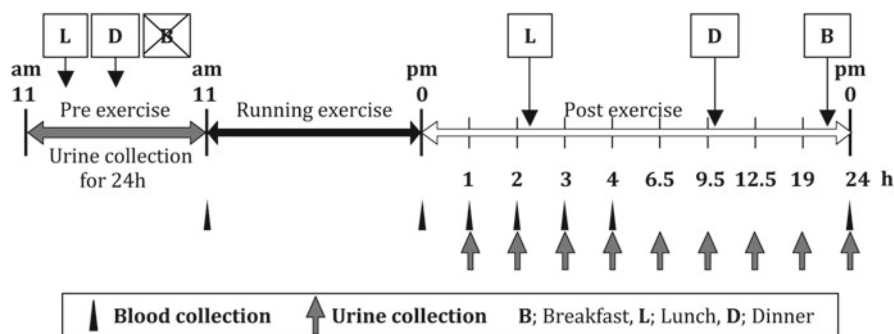

Fig. 1 Experimental protocol of the transient running exercise in a healthy subject. Urine was collected for 24 h before the exercise, and at 1, 2, 3, 4, 6.5, 9.5, 12.5, 19.5, and 24 h after the exercise. Blood was collected immediately before and after the exercise, and at 1, 2, 3, 4, and 24 h after the exercise. On the exercise day, subject did not take the breakfast, but took the lunch and dinner at 2.5 and 10 h after the exercise. Running speed was approximately 9 km/h

collected at 1, 2, 3, 4, 6.5, 9.5, 12.5, 19.5, and 24 h after the exercise. Furthermore, urinary volume in each collection was measured to calculate the amount of daily urinary excretion of taurine and NAT. Data of taurine and NAT in urine were expressed as the urinary excretion per hour. Taurine and NAT concentrations in serum and urine were quantified using HPLC-MS/MS system. Figure 1 Experimental protocol of the transient running exercise in a healthy subject. Urine was collected for 24 h before the exercise, and at 1, 2, 3, 4, 6.5, 9.5, 12.5, 19.5, and 24 h after the exercise. Blood was collected immediately before and after the exercise, and at 1, 2, 3, 4, and 24 h after the exercise. On the exercise day, subject did not take the breakfast, but took the lunch and dinner at 2.5 and 10 h after the exercise.

The study was carried out in accordance with the Declaration of Helsinki and was approved by the Human Subjects Committee of the Tokyo Medical University Ibaraki Medical Center.

2.3 NAT Production from Cultured Skeletal Muscle Cell Exposed to Acetate and Taurine

Mouse differentiable myoblast (C2C12) was purchased from ATCC (Manassas, VA). C2C12 cells were cultured with growth medium (GM; DMEM supplemented with 10 % fetal bovine serum) until confluent, and thereafter, the medium was switched to differentiation medium (DM; DMEM supplemented with 2 % horse serum) (Miyazaki et al. 2013). After differentiation, myotube was exposed to 20 mM taurine in GM for 24 h. Thereafter, the myotube was washed with PBS twice, and further exposed to 1 mM sodium acetate in GM for 24 h. After the incubations,

the culture medium and the myotube were collected to analyze NAT and taurine using HPLC-MS/MS system. Data are obtained from four independent experiments ($N=4$).

2.4 Quantification of Taurine and NAT

Taurine and NAT in serum, urine, culture cell, and culture medium were quantified by HPLC-LC/LC system according to the methods of Shi et al. (2012) and Johnson et al. (2011) with some modifications. In brief, NATs as standard and internal standard were synthesized from taurine (Wako Pure Chemical Industries, Osaka, Japan) and 2-aminoethane-d4 sulfonic acid (taurine-d4; C/D/N Isotopes Inc., Quebec, Canada), respectively, by reaction with acetic anhydride. Five microliter of serum and urine samples and 50 μL of cultured medium were mixed with 100 ng taurine-d4 and 1 ng NAT-d4 as internal standard in 50 μL of acetonitrile-water (19:1, v/v) in a microcentrifuge tube. In assay for culture medium, ten-times volumes of sample and acetonitrile-water were used. After centrifugation, the supernatant was evaporated to dryness at 80 °C under a nitrogen stream. The residue was redissolved in 60 μL of 0.1 % formic acid, and an aliquot (1 μL) was analyzed by HPLC-MS/MS system.

2.5 Statistic Analysis

Statistical significances were determined by unpaired Student's *t*-test or one-way ANOVA multiple comparison test. Data were expressed as the mean ± SEM. Differences were considered as statistically significant when the calculated P value was less than 0.05.

3 Results

3.1 NAT and Taurine Concentrations in Serum After Full-Marathon

NAT was detectable in the serum sample (3.2 ± 0.2 nM at before the full-marathon). Immediately after the marathon, serum NAT concentration was significantly increased (18.8 ± 2.4 nM, $P<0.01$ compared to the before). Taurine concentration in serum was also significantly increased immediately after the marathon race (30.9 ± 1.6 μM vs. 39.8 ± 1.8 μM before the marathon race, $P<0.01$). After 1 day, taurine and NAT concentrations in serum recovered to the levels before the marathon race (31.4 ± 1.1 μM and 3.1 ± 0.2 nM, in taurine and NAT, respectively).

3.2 NAT and Taurine Concentrations in Serum and Urine After a Transient Exercise

Figure 2 shows the changes over time of NAT and taurine concentrations in serum (**a**) and urine (**b**) before and after the transient running exercise in a subject. In serum, taurine concentration increased immediately after the exercise compared to that before the exercise, and further increased at 1 h later (Fig. 2a). Thereafter, serum taurine concentration decreased. Compared to before the exercise, NAT concentration in serum markedly increased immediately after the exercise. However, serum NAT concentration recovered to the normal level until 1 h later.

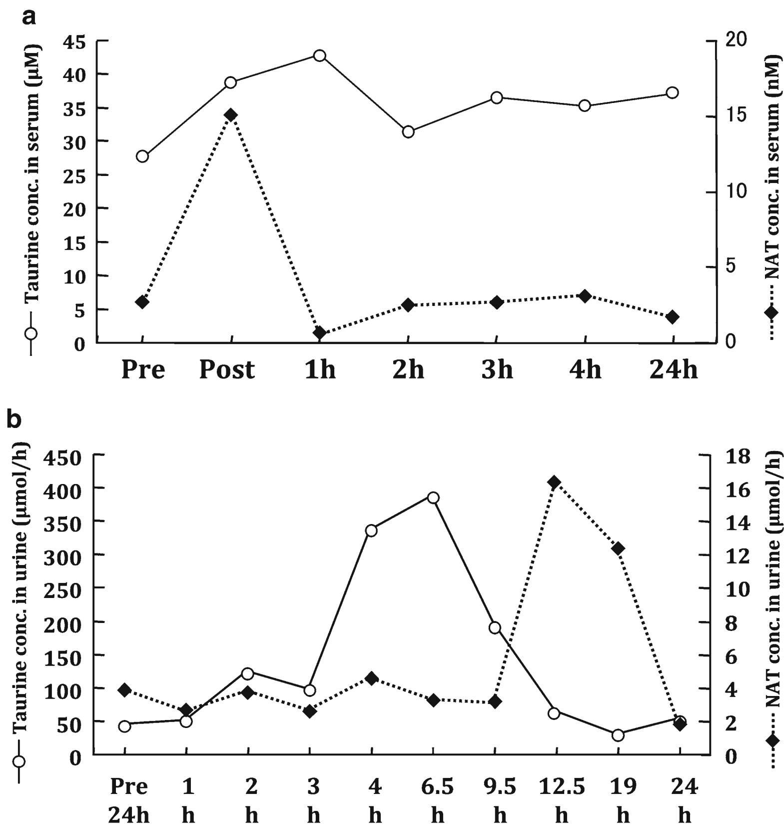

Fig. 2 Changes over time of taurine and NAT concentrations in serum and urine before and after a transient exercise. (**a**) Taurine and NAT concentrations in serum. (**b**) Taurine and NAT concentrations in urine. The concentrations in urine were expressed as the urinary excretion per unit time. *Pre* immediately before the exercise, *Post* immediately after the exercise, *Pre 24 h* 24 hours before the exercise, *conc.* concentration

Figure 2b demonstrates taurine and NAT excretions in urine expressed as its content per hour. In urine, there was little change in taurine concentration until 3 h later of the exercise, but markedly increased at 4 and 6.5 h later (Fig. 2b). Thereafter, the concentration gradually decreased, and returned to the level of before the exercise until 24 h later. NAT was also detectable in urine. Urinary NAT concentration unchanged until after 9.5 h of exercise. Thereafter, urinary NAT concentration dramatically increased between after 12.5 and 19 h of exercise, and returned to the level of before the exercise until 24 h later. Amount urinary excretions of both taurine and NAT increased after 24 h of the exercise compared to before 24 h (taurine; 0.97 mmol/day before exercise vs. 2.79 mmol/day after exercise, NAT; 91.4 μmol/day before exercise vs. 182.9 μmol/day after exercise).

3.3 Taurine and NAT Concentrations in Cell and Cultured Medium in the Myotube Exposed to Taurine and/or Acetate

Intracellular level of taurine was significantly increased by 20 mM taurine treatment for 24 h in both absent and present of acetate, while there was no significant difference between with and without acetate exposures regardless of taurine pretreatments (Fig. 3a). In cultured medium, taurine was undetectable in the conditions without taurine pretreatment (Fig. 3b). Taurine concentration in the cultured medium was detected after the taurine pretreatment, but there was no significant difference between with and without acetate exposures.

NAT could be detected in the both cell and cultured medium (Fig. 3c, d). Intracellular NAT level was significantly increased by 20 mM taurine pretreatment (Fig. 3c) in both with and without acetate exposures. In the taurine pretreated cells, intracellular NAT concentration was significantly higher in the 1 mM acetate exposure than in the absent of acetate. In the cultured medium, NAT was undetectable in the absent of taurine pretreatment (Fig. 3d). In the taurine pretreatment conditions, NAT concentration in the cultured medium was significantly higher in the acetate exposure than in the absent of acetate.

4 Discussion

In the present study, a novel metabolite NAT could be detected in human serum using HPLC-MS/MS system. Furthermore, the present study showed for first time that serum NAT concentration was significantly increased by the endurance exercise as full-marathon race, although blood level of taurine has been already known to be elevated after endurance exercises (Cuisinier et al. 2002; Ishikura et al. 2008). The significantly increased serum concentrations of both taurine and NAT returned to the level before the exercise by a day later.

Because Shi et al. has reported that urinary NAT concentration was increased during alcohol catabolism (Shi et al. 2012) to excrete the excess acetate into urine through *N*-acetylation of taurine, the resent study evaluated the effect of a transient endurance exercise on the urinary excretion of NAT. In the result, daily excretion of urinary NAT was significantly increased by the endurance exercise, suggesting that taurine might be acetylated with the elevated acetate during the exercise. The peak of urinary taurine excretion appeared between 4 and 6.5 h after the exercise. But, the peak of urinary NAT excretion was about 12 h later. There is a possibility that the urinary excretions of taurine and NAT might be influenced by taking meal, because

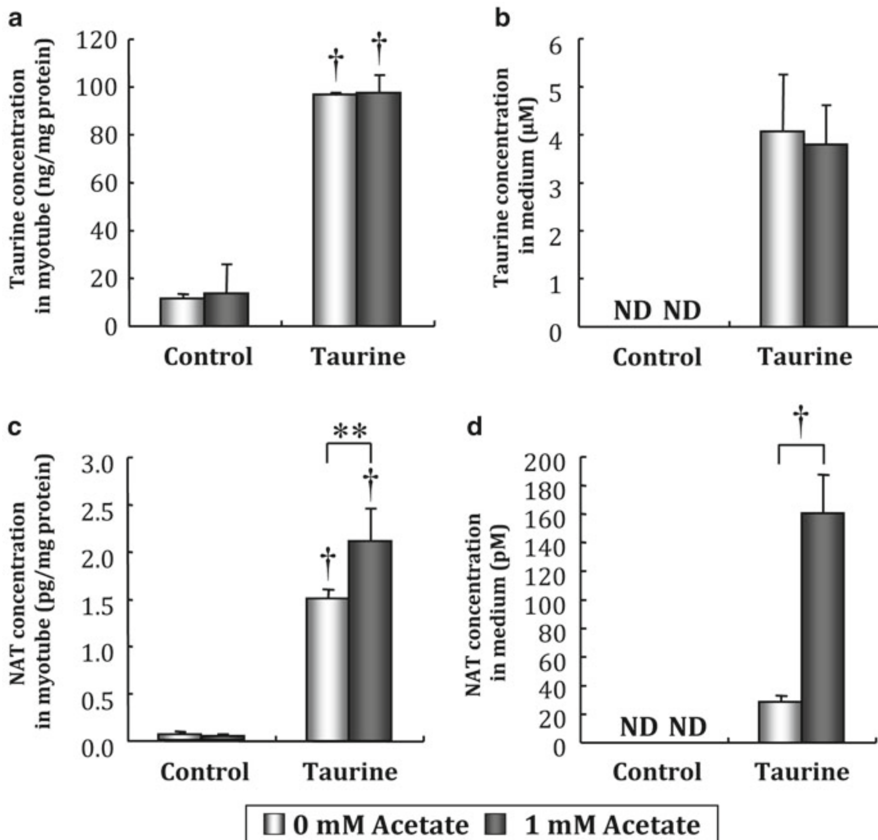

Fig. 3 Taurine and NAT concentrations in the myotube and cultured medium in the cultured skeletal muscle cell exposed to taurine and/or acetate. (**a**) Taurine concentration in the myotube, (**b**) Taurine concentration in the medium, (**c**) NAT concentration in the myotube, (**d**) NAT concentration in the medium. Control and Taurine show the 0 and 20 mM taurine in the culture medium, respectively, prior to the acetate exposure. Taurine and NAT concentrations in the myotube were expressed as per total protein content measured by the BCG method. Data are the mean ± SEM. *ND*; means "no detected". Symbols on the column without bar show the significant difference compared to the respective control. $^{**}P < 0.01$, $^{\dagger}P < 0.001$

the excretory peak of taurine was at after taking a lunch. However, the excretory peaks of taurine and NAT did not appear after taking a dinner and lunch, respectively, and the excretory peak of taurine to urine appeared after the peak of serum taurine after the exercise, and therefore, the changes of their excretions into urine is suggested to be influenced by the exercise rather than taking meals. It is unclear the reason why the timing of NAT excretion in urine was different with taurine, but there is a possibility of reaction time of acetylation with taurine and acetate.

Shi et al. described that *N*-acetylation of taurine is synthesized mainly in the liver and kidney during alcoholic catabolism (Shi et al. 2012). Because acetate produced in the liver would be used for acetyl-CoA synthesis as energy source through ACS2 in the skeletal muscle (Sakakibara et al. 2009), we hypothesized that *N*-acetylation of taurine might react in the skeletal muscle during endurance exercise. In the skeletal muscle cell culture experiment in the present study, NAT excretion from the myotube was significantly increased by exposing to taurine and acetate. Therefore, the increased NAT in serum after the endurance exercises might be derived from the skeletal muscle.

However, Shi et al. also mentioned that taurine would react directly with acetate in one-step enzymatic and ATP-independent pathway that does not require the conversion of acetate to acetyl-CoA, in the cytosol of kidney and liver during alcoholic catabolism (Shi et al. 2012). Indeed, acetyl-carnitine, a product after reaction with carnitine and excess acetyl-group of acetyl-CoA in β-oxidation (Liu et al. 2008), was unchanged in the both cell and medium by exposing to acetate (no data shown). Therefore, the production of NAT in the cultured muscle cell might be also due to the direct reaction of taurine with acetate, but not through acetyl-CoA. Furthermore, there is a possibility that NAT might be synthesized in other tissues including the kidney, and further studies are needed to clarify. Taurine has been reported to have a lot of biological, physiological, and pharmacological functions in various tissues including membrane stabilization (Pasantes et al. 1998), detoxification (Huxtable 1992; Miyazaki and Matsuzaki 2014), antioxidation (Nakamura et al. 1993; Miyazaki 2010), and osmoregulation (Nieminen et al. 1988). During the alcoholic catabolism, it is suggested that NAT might be produced in the kidney in order to facilitate the urinary excretion of excess acetate by higher hydrophilicity via reaction with taurine. On the other hand, the physiological role of the novel metabolite NAT has been unclear still now.

5 Conclusion

The present study confirmed (1): Serum NAT level significantly increased after the full-marathon race, (2): The increased NAT in serum recovered to the normal level after a day, (3): Urinary NAT excretion significantly increased after endurance running, (4): NAT production from the skeletal muscle cultured cell significantly increased following taurine and acetate treatments. These results indicate the significant increases of NAT in serum and urine after endurance exercises in humans, suggesting that taurine might be *N*-acetylated with excess acetate in the skeletal muscle during the exercise.

Acknowledgements This work was supported by Kakenhi grants Number (25750334).

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Effects of Taurine Depletion on Human Placental Syncytiotrophoblast Renewal and Susceptibility to Oxidative Stress

Michelle Desforges, Hannah Whittaker, Etaoin Farmer, Colin P. Sibley, and Susan L. Greenwood

Abbreviations

CTB	Cytotrophoblast
ETC	Electron transport chain
FGR	Fetal growth restriction
hCG	Human chorionic gonadotrophin
PE	Pre-eclampsia
ROS	Reactive oxygen species
STB	Syncytiotrophoblast
TauT	Taurine transporter

1 Introduction

Taurine (2-aminoethanesulfonic acid) is the most abundant free amino acid in the human placenta (Philipps et al. 1978). In syncytiotrophoblast (STB), the solute transporting epithelium of the placenta, the activity of the taurine transporter (TauT) in the maternal-facing microvillous membrane of the human placental syncytiotrophoblast (STB) is important to achieve a high intracellular taurine concentration and maintain a gradient that favours taurine efflux towards the fetus, where it is necessary for organogenesis (Sturman 1988; Han et al. 2000).

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We have previously reported reduced STB TauT activity in the pregnancy complication pre-eclampsia (PE) (Desforges et al. 2013a), a severe high blood pressure condition which is often accompanied by fetal growth restriction (FGR). In non-placental cells, taurine protects against damage caused by factors which are elevated in PE such as inflammatory cytokines and reactive oxygen species (ROS) (Wojtecka-Lukasik et al. 2006; Oriyanhan et al. 2005). Emerging evidence suggests that the cytoprotective role of taurine is related to its ability to promote mitochondrial function (Jong et al. 2010, 2012). Interestingly, mitochondrial dysfunction has been demonstrated in placentas from pregnancies complicated by PE and FGR (Muralimanoharan et al. 2012; Mando et al. 2014). Therefore, the reduction in STB taurine uptake in PE, in addition to restricting taurine efflux to the fetus, could compromise STB function and cytoprotection.

In the human placenta, renewal of STB is essential to preserve its function as a solute transporting epithelium and endocrine/paracrine organ, maintaining nutrient delivery to the fetus and producing hormones that sustain pregnancy. STB is renewed during pregnancy by a process of cellular turnover involving proliferation of the underlying cytotrophoblast cells (CTB) followed by differentiation, fusion, and incorporation of their nuclei into the STB (Huppertz et al. 2006; Heazell and Crocker 2008). In addition to supplying cellular energy, mitochondria are involved in a range of processes important for maintenance and function of STB, such as intracellular signalling, cellular differentiation, and cell death.

In PE and FGR there is reduced CTB fusion (Langbein et al. 2008; Vargas et al. 2011), and increased CTB apoptosis (Longtine et al. 2012) which leads to placental insufficiency. Using CTB *in vitro*, we showed that siRNA-mediated TauT knockdown reduced intracellular taurine, inhibited the differentiation and fusion of cells to form multinucleate syncytia, and increased susceptibility to TNF α-mediated apoptosis (Desforges et al. 2013b).

We hypothesise that the reduction in TauT activity and intracellular taurine in PE impairs STB renewal and lowers cytoprotection to damaging factors present in the maternal environment by compromising mitochondrial function. To test this hypothesis, STB renewal and susceptibility to oxidative stress was determined in the placental villous explant model following β-alanine-mediated intracellular taurine depletion. In separate studies using the BeWo choriocarcinoma cell line as a model of CTB, markers of mitochondrial function and oxidative stress were examined following β-alanine-mediated intracellular taurine depletion.

2 Methods

2.1 Intracellular Taurine Depletion in Placental Explants and Effect of Oxidative Stress

Human term placentas (38–40 weeks gestation) were obtained within 30 min of caesarean section from uncomplicated singleton pregnancies following written informed consent as approved by the Central Manchester Research Ethics Committee.

Placental villous fragments were dissected and maintained in explant culture for 7 days as described previously (Siman et al. 2001). Intracellular taurine depletion was achieved by incubating explants in the presence of 10 mM β alanine for the duration of culture. HPLC of homogenized explant tissue confirmed that 10 mM β alanine reduced intracellular taurine by ~95 % (data not shown). To study the effect of oxidative stress following intracellular taurine depletion, placental explants were treated with 1 mM H_2O_2 from day 5 of culture. Explant culture medium was collected daily to measure hCG, secreted by differentiated STB, using a commercially available ELISA (DRG Diagnostics, Germany) according to the manufactures' instructions. Routine IHC was used to detect cytokeratin 7 (an epithelial marker allowing assessment of STB regeneration) and 8-hydroxyguanosine (marker of oxidative DNA damage) in formalin-fixed wax-embedded explant tissue samples.

2.2 Effect of Intracellular Taurine Depletion on Markers of Mitochondrial Function in Trophoblast Cells

BeWo choriocarcinoma cells were incubated in the presence or absence of 5 mM β-alanine for 72 h before isolation of mitochondria using a mitochondria/cytosol fractionation kit (Abcam). Western blot analysis of subunits in mitochondrial electron transport chain (ETC) complex proteins I–V was then performed using a Total OXPHOS antibody cocktail (Abcam, 1:200 dilution). Nitrocellulose membranes were stripped and re-probed for VDAC (Cell signalling, 1:1,000 dilution). Developed film was scanned and the mean signal intensity of the immunoreactive species determined using Image J software. To account for any variability in sample loading, signal intensity of complex proteins I-V in each sample was normalised to the corresponding VDAC signal intensity.

Mitochondrial morphology and ROS in BeWo cells were assessed using MitoTracker® and CellRox® fluorescent probes respectively. To study the effect of oxidative stress following β-alanine-mediated intracellular taurine depletion, BeWo cells were treated for 1 h with 1 mM H_2O_2 prior to immunofluorescence.

2.3 Statistical Analysis

Paired data from placental villous explants were analysed by Wilcoxon matched pairs signed rank or Friedman test as appropriate. Western blotting data were analysed by Mann Whitney. A calculated p value of <0.05 was considered statistically significant using GraphPad Prism version 5.

3 Results

3.1 Intracellular Taurine Depletion Compromises STB Renewal

During placental villous explant culture, STB sheds over the first 2–3 days and thereafter is regenerated and maintained by processes of cellular turnover resembling those *in vivo*. STB regeneration *in vitro* is accompanied by increased hCG secretion into the explant medium (Siman et al. 2001; Audette et al. 2010). Cytokeratin 7 IHC allowed visualization of both the shed STB and newly regenerated STB (Fig. 1a). STB regeneration on day 7 of culture was assessed by

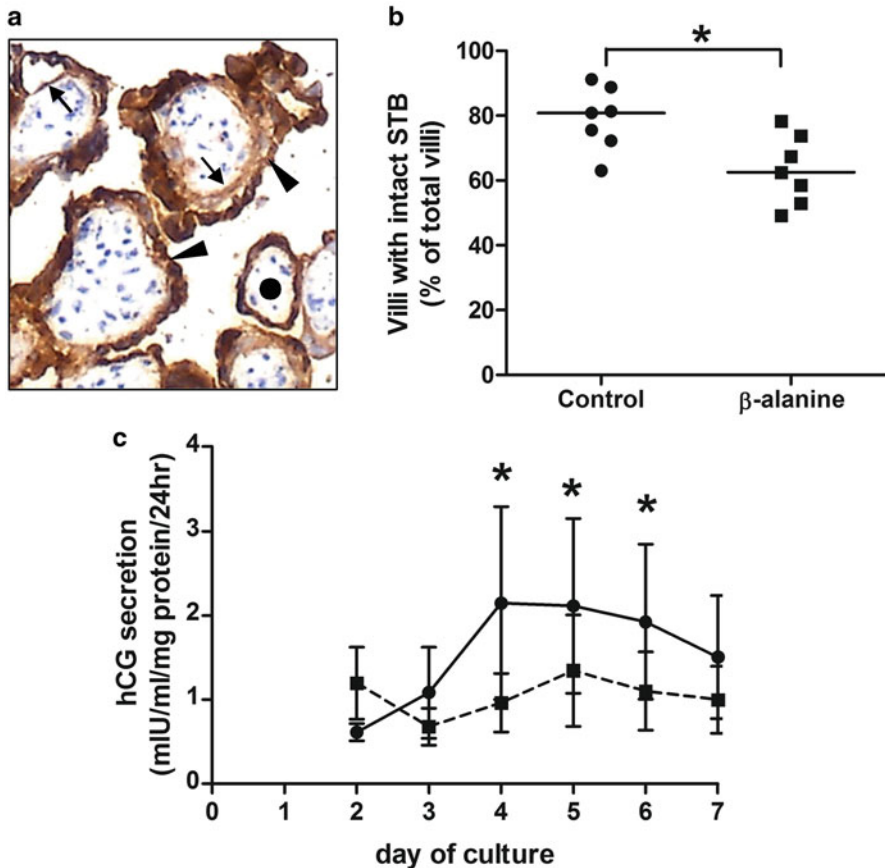


Fig 1 Analysis of STB renewal in placental villous explants. (**a**) Cytokeratin 7 IHC in explants fixed on day 7 of culture for analysis of STB shedding (*arrowheads*) and renewal (*arrows*). *Circle* indicates an example of a villus with intact STB. (**b**) Proportion of villi with intact STB (n = 7; *line* represents median). (**c**) Time course of hCG secretion (mean ± SE) by control (*circles*) and β-alanine-treated (*squares*) explants (n = 7). *$p < 0.05$, Wilcoxon matched pairs signed rank

expressing the number of villi with intact STB as a proportion of the total number of villi within a field of view. In control explants, complete STB regeneration was evident in 60–90 % of villi (Fig. 1b). In explants with intracellular taurine depletion, complete STB regeneration was significantly reduced compared to controls (Fig. 1b). As previously described (Siman et al. 2001; Audette et al. 2010), hCG secretion by control explants increased from day 2 to day 4 of culture (Fig. 1c). Intracellular taurine depletion with β-alanine significantly reduced hCG secretion compared to matched controls (Fig. 1c).

3.2 *Intracellular Taurine Depletion Increases STB Susceptibility to Oxidative Stress In Vitro*

IHC to detect 8-hydroxyguanosine (marker of oxidative DNA damage) in STB nuclei and cytoplasm (i.e. mitochondrial DNA) (Fig. 2a, b respectively) was performed in placental villous explants following chronic treatment (48 h) with 1 mM H_2O_2. Semi-quantitative analysis of staining revealed H_2O_2 treatment alone was insufficient to induce oxidative damage to either nuclear DNA or mitochondrial DNA in STB (Fig. 2c, d respectively). However, oxidative damage to both STB nuclear DNA (Fig. 2c) and mitochondrial DNA (Fig. 2d) was significantly higher in explants treated with H_2O_2 following intracellular taurine depletion with β-alanine when compared to control, H_2O_2 or β-alanine alone.

3.3 *Evidence of Mitochondrial Dysfunction in BeWo Cells Following Intracellular Taurine Depletion*

Western blot analysis of mitochondrial ETC complex proteins I–V in isolated mitochondria from BeWo cells (Fig. 3a) demonstrated significantly reduced expression of ETC complex V (ATP synthase) subunit in cells with β-alanine-mediated taurine depletion (range: 0.72–0.9) compared to controls (range: 0.75–2.5) (Fig. 3b).

Immunofluorescence to detect active mitochondria (Mitotracker®) and ROS (CellRox®) in BeWo cells demonstrated that under control conditions, mitochondria were diffusely spread throughout the cell and levels of ROS were minimal (Fig. 4; Control). Taurine depletion induced mitochondrial swelling and fragmentation of the diffuse mitochondrial network (Fig. 4; β-alanine). In addition, there was an increase in ROS which co-localised with the nuclei and mitochondria. A similar alteration to mitochondrial morphology was seen in positive control cells incubated with 30 μM antimycin A (an inhibitor of ETC complex III) for 1 h: mitochondria appeared swollen and clustered around cell nuclei (Fig. 4; Antimycin A). There was no apparent increase in ROS following H_2O_2 treatment of control cells (Fig. 4; H_2O_2). However, in cells with β-alanine-mediated taurine depletion, H_2O_2 treatment increased levels of ROS co-localised with nuclei and some mitochondria and also increased ROS in the cytoplasm.

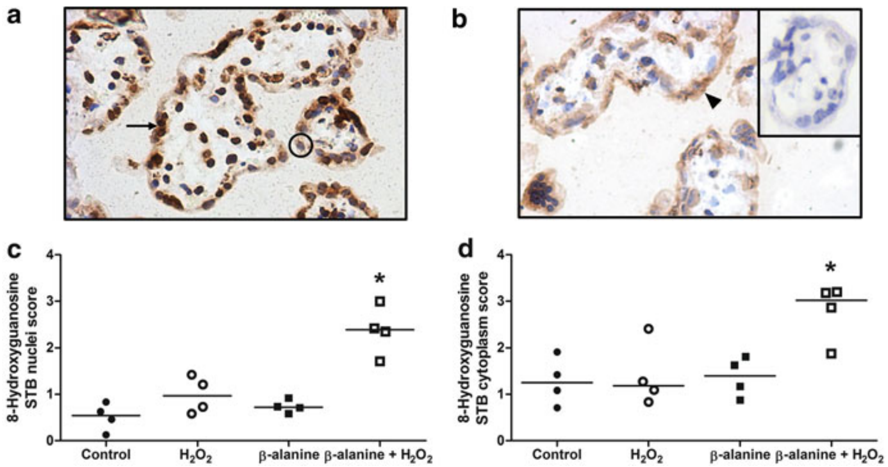
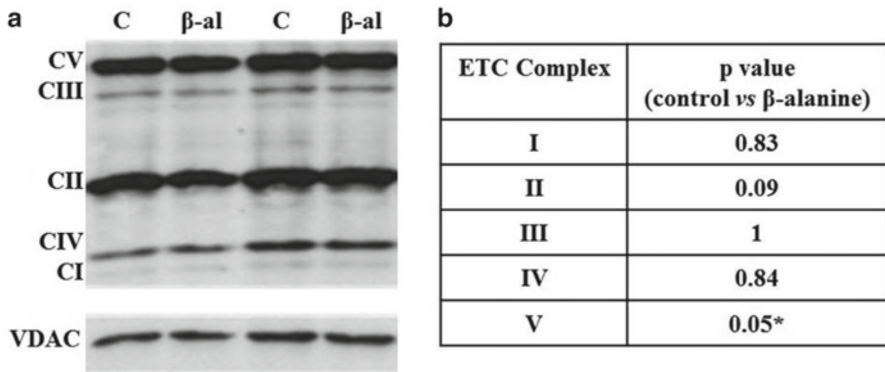


Fig. 2 IHC for oxidative DNA damage in STB following β-alanine-mediated taurine depletion and H_2O_2 treatment. Detection of oxidative damage to DNA in (**a**) the nucleus (arrow = +ve nuclei, circle = −ve nucleus) and (**b**) the cytoplasm (mitochondrial DNA; indicated by *arrowhead*) using positive staining for 8-hydroxyguanosine (*brown*). Counterstained with hematoxylin. *Inset*: negative control (non-immune IgG replaced primary antibody). (**c**, **d**) Semi-quantitative assessment of 8-hydroxyguanosine staining in STB using a scale of 0–4 (n = 4; *line* represents the median. *$p < 0.05$, Friedman with Dunn's post test)



ETC Complex	p value (control *vs* β-alanine)
I	0.83
II	0.09
III	1
IV	0.84
V	0.05*

Fig. 3 Western blot analysis of mitochondrial ETC proteins in BeWo cells following β-alanine-mediated taurine depletion. (**a**) Representative Western blot of complex I–V (CI–CV) subunit expression in control (C) and β-alanine-treated cells (β-ala). VDAC was used as a loading control. (**b**) Summary table of p values following comparison of subunit expression, normalized to VDAC, in control and β-alanine treated cells. n = 4/5; *$p < 0.05$, Mann Whitney

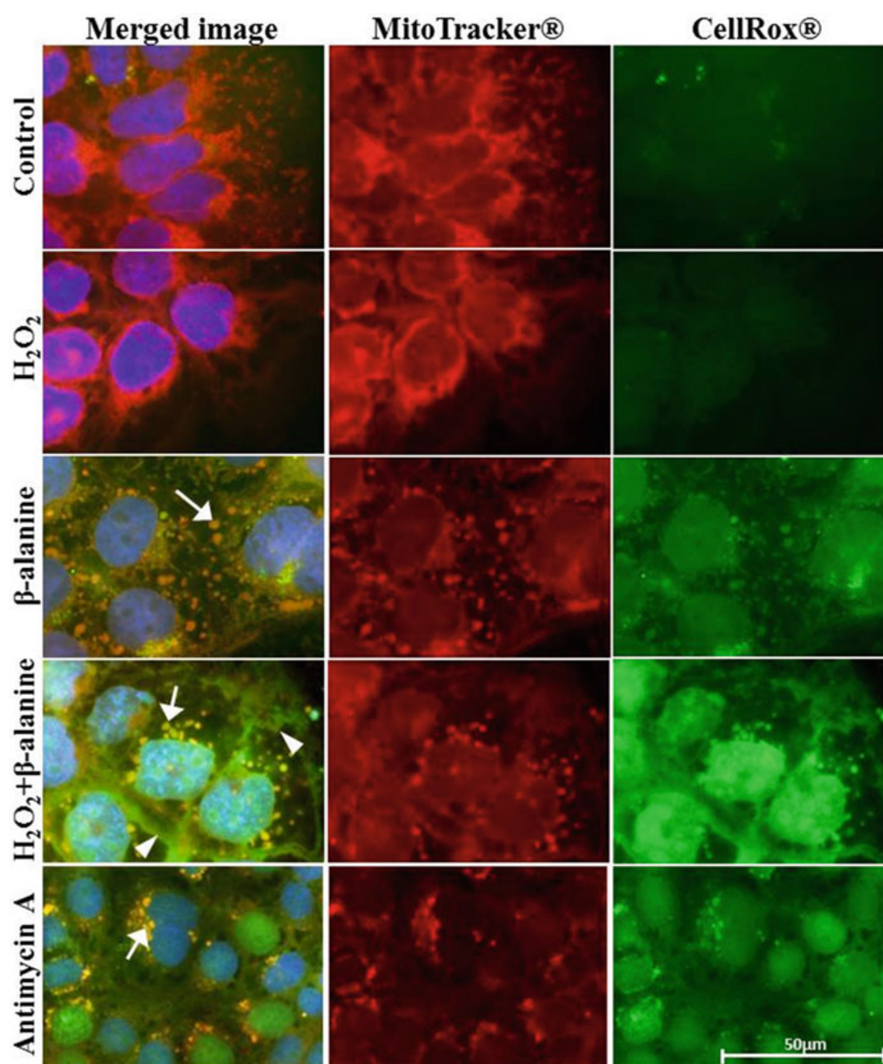


Fig. 4 Mitochondrial morphology and ROS in BeWo cells following β-alanine-mediated taurine depletion and H_2O_2 treatment. CellRox® (*green*) to detect ROS and MitoTracker® (*red*) to detect mitochondria. Merged image with DAPI nuclear counterstain (*blue*). ROS can be seen in the cytoplasm (*arrowheads*) and co-localised with swollen mitochondria (*arrows*) and nuclei. Antimycin A included as a positive control. All images were captured at the same exposure to allow comparison of fluorescence intensity. Representative images of n = 6

4 Discussion

In previous studies we have demonstrated compromised CTB differentiation *in vitro* following intracellular taurine depletion (Desforges et al. 2013b). Differentiation of CTB is a key event in the process of STB turnover *in situ*, preceding CTB fusion with STB and the subsequent incorporation of fresh cellular material. Here we have provided further evidence that intracellular taurine is necessary for maintenance of STB by demonstrating impaired renewal of STB in placental villous explants following intracellular taurine depletion.

Dysregulated STB turnover is evident in several pregnancy complications and in PE it arises from reduced CTB fusion (Langbein et al. 2008; Vargas et al. 2011) and increased CTB apoptosis (Longtine et al. 2012). The etiology of PE is poorly understood but the disease is thought to arise from partial failure of placentation, leading to compromised uteroplacental blood flow and ischemia-reperfusion injury of the placenta (Huppertz 2008). This can induce both local and systemic oxidative stress, defined as an imbalance between ROS and antioxidant capacity. Indeed, numerous studies have shown that women with PE have increased circulating markers of oxidative stress and reduced antioxidant capacity (Siddiqui et al. 2010; Hubel 1999) along with increased oxidative stress in the placenta (Myatt and Cui 2004). *In vitro* experiments show that ROS damage the placenta through effects on STB turnover (Moll et al. 2007). Elevated ROS and reduced placental TauT activity in PE are therefore likely contributors to disrupted STB turnover associated with this pregnancy complication.

Our data also demonstrate the importance of intracellular taurine in STB/CTB for protection against ROS. ROS can react rapidly with DNA, proteins and lipids, thereby leading to oxidative damage, which impairs cellular function (Burton and Jauniaux 2011). Here we have shown that intracellular taurine depletion in placental villous explants rendered STB more vulnerable to oxidative DNA damage following treatment with H_2O_2. In experiments with taurine deplete BeWo cells, H_2O_2 treatment was associated with increased ROS in the cytoplasm. Interestingly, in control (i.e. taurine replete) cells and explant tissue, H_2O_2 treatment did not increase intracellular levels of ROS or induce oxidative DNA damage. There is some evidence that taurine functions as a free radical scavenger (Cheong et al. 2013), but there is little evidence that it upregulates the antioxidant defences of the cell. When cells are subjected to oxidative stress, mitochondria are capable of drawing upon their reserve capacity to serve the increasing energy demands for maintenance of organ function, cellular repair, or ROS detoxification. The final outcome is determined by a balance that may lean toward either apoptosis or cellular recovery. We did not explore apoptosis in the current study following intracellular taurine depletion and H_2O_2 treatment. However, in a previous study we found no effect of intracellular taurine depletion on basal levels of apoptosis in STB, but there was increased susceptibility to apoptotic cell death following treatment with the inflammatory cytokine TNFα (Desforges et al. 2013b). Collectively, these data indicate a cytoprotective role for taurine in STB similar to that reported for other non-placental cells (Wojtecka-Lukasik et al. 2006; Oriyanhan et al. 2005).

In non-placental cells, taurine is required for efficient translation of proteins in the ETC (Jong et al. 2010, 2012). Intracellular taurine is therefore important for maintaining ETC activity and enhancing mitochondrial reserve capacity. Biochemical modelling has also demonstrated an important role for taurine as a mitochondrial matrix buffer, stabilising oxidative metabolism (Hansen et al. 2006). These observations suggest the mechanism underlying the antioxidant activity of taurine is related to its role in promoting mitochondrial function. In the current study there were signs of mitochondrial dysfunction in taurine deplete BeWo cells evidenced by reduced expression of ETC complex V subunit (responsible for generation of ATP via oxidative phosphorylation), swelling of mitochondria, and fragmentation of the normally diffuse mitochondrial network. There was also increased ROS co-localising with the nuclei and mitochondria indicating a higher level of oxidative stress. This is the first evidence to suggest taurine has a role in the regulation of normal mitochondrial function in placenta. We speculate that the increased susceptibility of taurine deplete STB/CTB to damage following H_2O_2 treatment that we observed is related to mitochondrial dysfunction and insufficient capacity to detoxify accumulating ROS in these cells.

Our *in vitro* data suggest reduced placental taurine transport in PE could compromise placental development and increase CTB/STB susceptibility to death and damage through an effect on mitochondrial function. Structural, functional, and genetic changes in mitochondria have been reported in STB from patients with PE. For example, the mitochondria show swelling with a loss of cristae (Muralimanoharan et al. 2012), there is a reduction in the expression and activity of ETC complexes I and III and the ETC enzyme, cytochrome c oxidase (Muralimanoharan et al. 2012; Furui et al. 1994; Matsubara et al. 1997), and mutations in mitochondrial tRNA genes have been shown in two families with a high occurrence of PE (Folgero et al. 1996). It has yet to be determined whether these observations are contributing factors to the disease, or a consequence of ongoing oxidative stress which leads to mtDNA and/or protein damage.

5 Conclusion

In summary, these *in vitro* studies showed that intracellular taurine depletion in the placenta compromised STB regeneration and resulted in an increased susceptibility to oxidative stress, evidenced by nuclear and mitochondrial DNA damage. Intracellular taurine depletion was also associated with markers of mitochondrial dysfunction and increased ROS in trophoblast cells. The effects of taurine deficiency *in vitro* are consistent with dysregulated STB renewal, elevated oxidative stress and mitochondrial dysfunction that are features of placental pathology in PE. Reduced placental taurine uptake in PE could therefore be a contributing factor to the placental insufficiency associated with this pregnancy complication.

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In Vitro Analysis of Taurine as Anti-stress Agent in Tomato (*Solanum Lycopersicum*)-Preliminary Study

Dong-Hee Lee

Abbreviations

CLIM	Callus induction media
FW	Fresh weight
IAA	Indoleacetic acid
Lyso-PC	Lysophosphatidylcholine
SIM	Shoot induction media

1 Introduction

Taurine is an amino acid that exists predominantly in animal cells. It is abundant in eggs, fish, meat, and most dairy products. Plant tissues and cells, however, have little or no taurine content, although some taurine can be found in red algae. This fact strongly indicates that plants have lost their ability to synthesize and utilize taurine during the course of evolution. Since effects of taurine on plants have not been studied extensively, its potential function is currently unknown. A recent report indicated that providing additional nutritional support in the form of amino acids might help to increase plant growth (George et al 2008; Zoltan et al. 2011).

In living organisms, taurine requires a special type of transporter to be initially internalized into cells. Taurine is expected to minimally cross biological membranes due to its highly hydrophilic nature, and plants do not have the necessary transporters. Some plant extracts even have an inhibitory component, lysophosphatidylcholine

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(Lyso-PC), which has been shown to impede the uptake of taurine in human intestinal epithelial cells (Ishizuka et al. 2000). The main inhibitory mechanism is the specific blocking of taurine transporters on the cell membrane. Since Lyso-PC did not obstruct the uptake of other amino acids such as leucine and glutamic acid, the material appears to exclusively target the taurine transporter and its uptake.

In comparison to animals, plants have developed more powerful anti-stress machinery and chemical resources for various environmental pressures due to their lack of mobility (Foyer and Noctor 2005; Gill and Tuteja 2010). However, these resources are difficult to characterize using a field study. Plant tissue culture refers to the proliferation of cells, tissues, and organs from an intact plant on solid or in liquid media under sterile and controlled settings (Chatzissavvidis et al. 2008; Garg 2010; Gaspar et al. 2002; Joyce et al. 2003). This technique provides plant scientists the opportunity to study the exclusive and multifaceted resources that plants have for coping with environmental stresses. In addition, plant tissue culture protocols allow to study the effects of a material in plant tissue without the concerns of absorption and transport (Neuman et al. 2009). Any chemical or organic factor can be absorbed into plant cells cultured in vitro without difficulty (Lokhande et al. 2011).

This technique is one of the key tools of plant biotechnology, especially for understanding the totipotent nature of plant cells. In addition, this technique allows for the study of large plant populations, stress treatment of large populations in a limited space and over a short period of time, and the homogeneity of stressor application (Sakthivelu et al. 2008; Lokhande et al. 2010). Because of these advantages, plant tissue culture techniques have vast potential for various applications for both plant science and commercial development, such as producing large numbers of identical individuals via micropropagation (George et al. 2008; Safarnejad 2004). Most importantly, plant tissue culture methods can be used as a model for inducing stress via different stress agents, studying a response of a plant to abiotic stresses, and even selecting strains that resist stress induced by salt or an insufficient supply of water (Gallego et al. 2002; Gandonou et al. 2006; Sen and Alikamanoglu 2011).

Amino acids are absorbed easily and have been shown to exert positive effects on plant tissues during plant tissue culture experiments. Taurine is also absorbed by plant tissue cultured in vitro. When amino acids were added to the tissue culture media, they significantly stimulated the growth of plants. Indeed, glutamate and aspartate increased the growth of tobacco calluses. This is because amino acids exert positive effects on cell division, colony growth, and plantlet differentiation. The positive effects of amino acids are highly consistent throughout shoot induction and organogenesis. Sporadic shoot development increased in cauliflower explants grown in media containing a mixture of amino acids (asparagine, proline, tyrosine, and phenylalanine). The reinforcement of amino acids can enhance morphogenesis, where they serve as a source of reduced nitrogen or as a complementary supply for nitrate. Likewise, L-methionine, a precursor of taurine or ethylene, promotes cytokinin activity and prompts the propagation of *Prunus glandulosa* var. sinensis cultured in media supplemented with taurine (Druart 1988; Ferreira and Lima-Costa 2006).

Table 1 Taurine content in plants

Species	Taurine content (mg/kg)
Rhodophyta (red algae)	
Agar (*Gelidium subcostatum*)	125 ± 1.96
Phaeophyta (brown algae)	
Kombu (*Laminaria japonica*)	16.6 ± 0.004
Sargassum (*Sargassum fulvellum*)	6.4 ± 0.287
Chlorophyta (green algae)	
Green sea fingers (*Codium fragile*)	1.89 ± 0.003
Bryophyta (mosses)	
Hair moss (*Polytrichum juniperium*)	0.16 ± 0.009
Pteridophyta (vascular plants)	
Lady fern (*Athyrium niponicum*)	0.22 ± 0.014
Spermatophyta (seed plants)	
Garden parsley (*Petroselium crispum*)	ND
Green onion (*Allium fistulosum*)	ND
Tomato (*Solanum lycopersicum*)	ND

Compiled from the data of Kataoka and Ohnishi (1986) and Spitze et al. (2003)

ND non detected

In order to characterize effect of taurineon plant stress, this study utilized plant tissue culture methods in tomato explants (*Solanum lycopersicum*), which were deficient in taurine like most higher plants (Table 1). This taurine deficiency is helpful for characterizing the novel function of taurine by incorporating it into the tissues of the tomato plant via tissue culture procedures. The explants were subjected to stress conditions and further treated with taurine. The anti-stress function of taurine was initially assessed basing on several vital signs: fresh weight, time to callus/shoot formation, and the standardized shoot number per callus. In another set of experiments, calluses were induced under normal conditions and subjected to stress before an extended application of taurine. In the latter case, the number of shoots per callus was compared between the taurine-treated and taurine-free groups.

2 Methods

2.1 Plant Materials and Culture Conditions

Tissue culture was performed according to a general culture procedure. Seeds of the tomato plant (*Solanum lycopersicum* var. 883) were sterilized in 1 % sodium hypochlorite for 10 min, and washed three times with sterile water. Seeds were planted

in a mixture of perlite and vermiculite (1:1) and moisturized daily with distilled water. Explants were prepared from pieces of leaves at the 4–5 stage following disinfection with 10 % hypochlorite for 10 min, and then with 95 % ethanol for 30 s. They were rinsed copiously with deionized water. Explants were cultured on Murashige and Skoog (MS) medium, which was supplemented with 30 g sucrose and solidified with agar at 7 g/L (Murashige and Skoog 1962). The pH of medium was adjusted to 5.8.

2.2 Stress Induction and Taurine Treatment

To induce stress, explants were subjected to hydrogen peroxide or NaCl. They were soaked in a solution with hydrogen peroxide (25 %, v/v) or NaCl (20 %, w/v) for 30 min. After the application of the above stress factors, the calluses were induced in callus induction media (CLIM). The CLIM was prepared by supplementing the MS media with three plant hormones: indoleacetic acid (IAA), alpha-naphthalene acetic acid, and kinetin at 1, 1, and 2 mg/L, respectively. To prepare the taurine media, taurine was added to the media to the final concentrations of 5 and 10 mg/mL. A no-stress control was cultured in a taurine free CLIM. The cultures were maintained under normal growth conditions (16/8 h light/dark periods). The initiation of callus formation was recorded daily.

2.3 Induction of Callus and Shoot

For the experiments on shoot induction and FW measurement, calluses were induced under stress-free conditions, that is, explants were cultured in CLIM, without being treated with NaCl or hydrogen peroxide, to obtain enough calluses for the two categories of experiments. Calluses were separated into two groups: one for FW measurement and the other for the shoot-induction experiment. For the FW measurements, calluses were further cultured in CLIM after being treated with hydrogen peroxide or NaCl for 30 min. They were subcultured separately into the two culture groups in the media with or without taurine . Calluses were weighed after the moisture was removed by spotting them on a piece of sterile filter paper. Shoots were induced by placing the calluses in shoot induction media (SIM) after treatment with hydrogen peroxide or NaCl for 30 min. Following stress application, the calluses were further subdivided into two different culture groups: taurine or no taurine. The SIM was prepared by adding zeatin (1.0 mg/L) and IAA (0.1 mg/L) to the media. Data were collected in terms of the time taken to shoot formation and the number of shoots per leaf disk. The number of shoots was counted under a colony counting magnifier (10×).

3 Results

This study utilized plant tissue culture protocols that facilitated taurine transport in the tomato plant tissues independent of the whole plant structure in order to investigate whether taurine can alleviate stress induced by salt and hydrogen peroxide in tomato tissues. Various vital markers were used to characterize function of taurine as a potential anti-stress agent, namely the duration to callus/shoot formation, number of shoots per leaf disk, and FW. Under the stress conditions, tomato explants showed the typical symptoms. Callus induction and shoot formation were significantly delayed or inhibited and most of the explants even failed to survive after the stress treatment. After the taurine treatments, however, the tomato tissues appeared to recover from the induced stress in terms of both the explants and calluses. In terms of the vital markers, tomato tissues responded positively upon treatment with taurine. Specifically, when treated with taurine, the explants or calluses appeared to significantly recover from the stresses induced by NaCl and hydrogen peroxide.

3.1 Taurine Supplementation Helps Explants to Recover from Stress

When the FWs were measured, taurine-treated calluses gained more weight compared to the calluses that were not treated with taurine after the stress induction. A different degree of callus development was apparent between the taurine-treated calluses and the taurine-free controls. When the explants were subjected to hydrogen peroxide or NaCl, the majority of explants suffered from the stress in the absence of taurine. Most leaf disks formed only a few calluses and they deteriorated loosing their original structure. This was very different from the responses of their taurine-treated counterparts. Taurine treatment helped explants to form calluses, as shown in Fig. 1a. Of the explants used for comparison, more than 20 % formed calluses; however, minimal explants formed calluses in the absence of taurine. The percentage of callus formation appeared to increase in a concentration-dependent manner. These results strongly indicate that taurine plays an important role in recovering from stress (Fig. 1b).

3.2 Taurine Increases the Size of Calluses Grown Under Stress

Effect of taurine was studied on calluses that were undergoing shoot development. After the application of two stress inducers were applied to the calluses, shoot development was significantly affected. When the calluses were pre-treated with NaCl or hydrogen peroxide, shoot formation was evidently delayed and

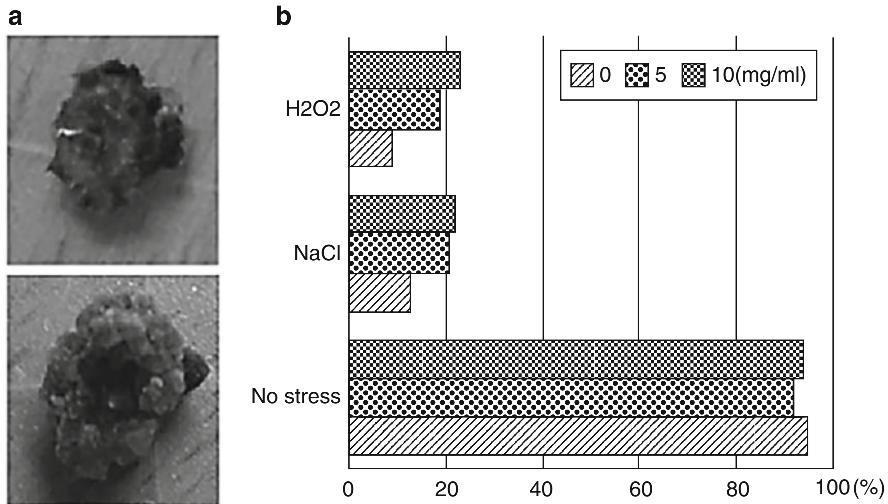

Fig. 1 Effect of taurine on callus development under stress conditions. (**a**) Explants were treated with hydrogen peroxide (H_2O_2). Most explants did not survive the stressful effects of hydrogen peroxide as shown in the *top panel*. When treated with taurine, however, explants formed a few calluses as shown at the *bottom*. (**b**) Explants responded positively to the taurine treatment. The responsiveness is expressed in terms of the percentage of callus formation from the leaf explants. The effect of taurine on callus formation increased in a dose-dependent manner

inhibited. However, the taurine-treated group was less severely affected, as this group showed mild reductions in the number of shoots per callus and the time to shoot formation.

FW measurement in a group of calluses was performed. Calluses were further cultured in CLIM after the stress treatments. After the calluses were treated with the two stress factors, they failed to gain any significant weight in the absence of taurine (Fig. 2). Their weight was significantly affected by the stress agents. In contrast, the taurine-treated group showed a significant recovery from the stress, as far as the gain in FW was concerned.

3.3 Taurine Alleviates Delayed Onset in Shoot Development

Calluses were treated with shoot inducing agents. When the calluses began to form shoots and the three stress factors were applied to the explants, they were significantly affected by the stress agents. However, the effect of the stress on the taurine-treated group (10 mg/mL) was less severe in terms of the reduction in days to shoot formation (Fig. 3).

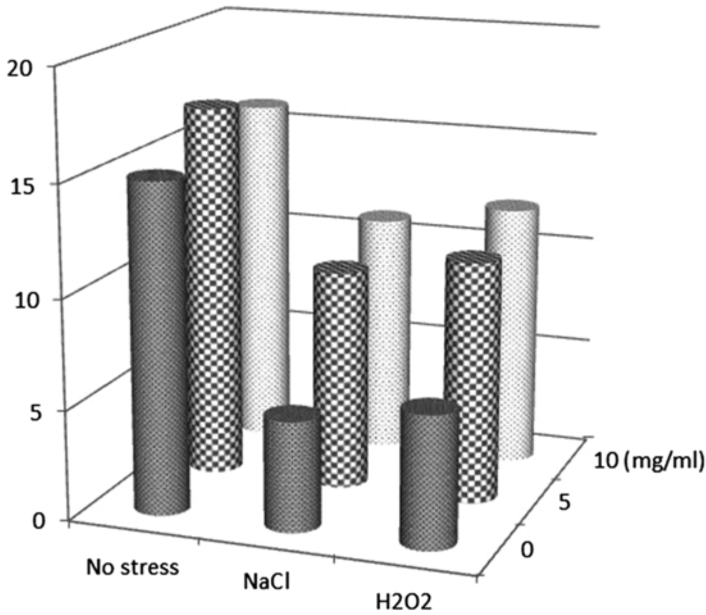

Fig. 2 Comparison of the FW of calluses. Calluses were cultured in callus inducing media. The FW was measured twice: at the beginning and at the termination of callus culture. FW increments are expressed as in mg in the graph

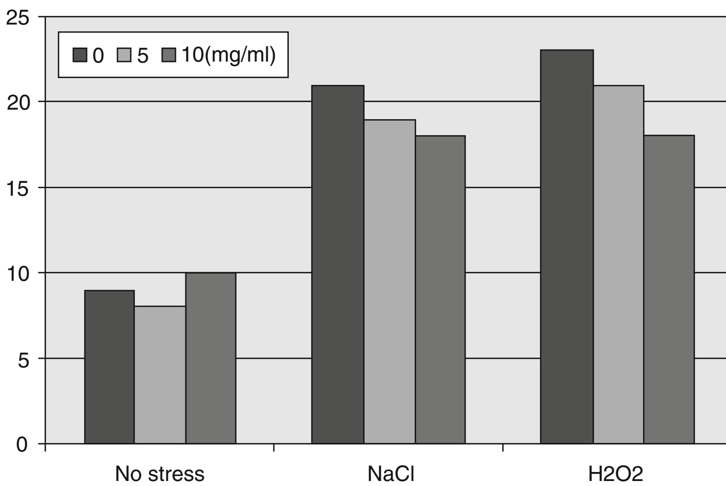

Fig. 3 Time (days) to shoot formation. Stress-induced explants were cultured in taurine-containing media with concentrations of 0, 5, and 10 mg/mL. Explants that were not exposed to the stress began to form calluses within 15 days of the culture. Most of the stressed explants showed a significant delay in callus formation. Taurine appeared to shorten the time needed for callus formation on the stressed plants

3.4 Taurine Increases the Number of Shoots per Callus Developing Under Stress

Calluses were subjected to stress induction and were incubated in taurine-containing media. The stressed calluses developed fewer shoots than the stress-free controls. At the end of the shoot-induction experiment, the final number of shoots was counted per callus and then averaged (n=5). A significant reduction in the number of shoots per explant was evident in the stressed calluses (Fig. 4). For calluses in the taurine-treated media, however, the calluses recuperated from the reduction in shoots formation.

The tomato tissues appeared to recover from the induced stress. When treated with taurine, calluses appeared to recover from the stresses induced by NaCl and hydrogen peroxide. The vital tissue markers used in the present study consistently indicated a role of taurine as a potential anti-stress agent. Callus induction and shoot formation were greatly improved after taurine treatment along with increases in the FW. These data indicate that taurine may serve as a potent anti-stress agent in plant cells, as well as in animal cells.

4 Discussion

When cultured in vitro, tomato tissues were very susceptible to the two stress factors: NaCl and hydrogen peroxide. Although tomato explants and calluses showed typical stress symptoms after treatment with the stressors, the symptoms did not

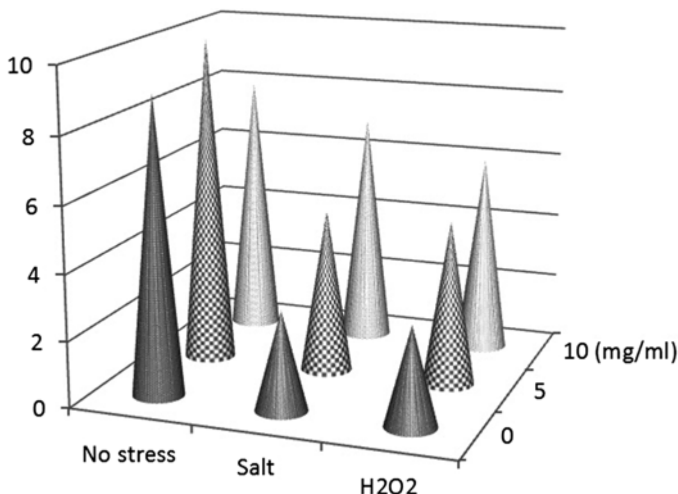

Fig. 4 Shoot regeneration comparison. Stress-induced calluses were cultured in taurine-containing media as in Fig. 3. The stress-free calluses made shoots within a month. The stressed calluses showed a reduction in the number of shoots per explant. When treated with taurine, however, the calluses appear to recover from this reduction in terms of the number of shoots formed

appear when tomato tissues were treated with taurine after the induction of stress. They appeared to recover from the stress upon treatment with taurine. After the stress application, callus induction and shoot formation were significantly delayed in comparison to the stress-free controls. In the presence of taurine, however, tomato tissues appeared to recover from the stress symptoms. Taurine may counteract the effects of stress caused by hydrogen peroxide and NaCl in a dose-dependent manner in tomato cells cultured in vitro. The reduced number of shoots in each stress condition can be restored by taurine. The time for callus formation decreased after taurine treatment under the stress conditions. This evidence indicates that taurine can serve as a potent anti-stress agent in plant cells.

Considering that tomatoes lack the relevant transporters for taurine, it is almost impossible to determine whether taurine can restore the well-being of tomato cells or tissues that undergo stress. This study utilized plant tissue culture protocols that circumvent the potential problems in taurine transport. In addition, plant tissue culture is important for devising an efficient protocol for callus proliferation to start in vitro selection for various stress tolerance, and to expand the opportunities for genetic manipulation of useful plants through tissue culture using various explants and media (Jha and Ghosha 2005; Hossain et al. 2007; Lu et al. 2007). Tissue culture serves as a useful tool for characterizing the effect of a biochemical substance on plants by a direct application. It can overcome most problems regarding internal transport. The results of this study indicated that taurine showed great anti-stress potential, which may further help plants to survive under stress if they can easily internalize taurine.

Throughout this study, taurine was shown to be a competent and useful anti-stress agent. If added to plant growth media or fertilizer, it may help the plants to gain anti-stress abilities. According to Hao et al. (2004), taurine may be utilized in a whole plant, as taurine was able to promote plant growth in wheat seedlings. Additional studies may be necessary to establish how taurine can be utilized in plants despite not having any pertinent transporters. Most plants lack taurine as a cellular component, although lower plants may still contain taurine, especially marine algae (red algae). Within green plants, it is likely that taurine has been deleted during the course of evolution. Photosynthetic plants might have needed another type of anti-oxidant or anti-stress agent. Studies on the compounds able to replace taurine as anti-stress agents in higher plants and on the reason of disappearance of taurine in the course of evolution may be very desirable.

This may be the first study to analyze potential of taurine as an anti-stress agent in plant tissue. Higher plants do not have taurine, and its potential function is currently unknown. Using a taurine-free background, this study revealed that taurine can serve as an anti-stress agent if added into plant tissues. Further studies may be necessary to investigate other potential functions under various conditions, for instance, ultraviolet irradiation and endoplasmic reticulum stress. Additional experiments may be required to study effects of taurine in an intact plant; however, taurine is not absorbed across biological membranes easily due to its hydrophilic nature. Some interdisciplinary efforts may be required to develop taurine derivatives that can be transported into plant tissues or cells to overcome the lack of taurine transporters in higher plants, especially seed plants.

5 Conclusion

Although tomato tissue was very susceptible to the stress factors (NaCl and hydrogen peroxide), it showed a resistance to the stress in the presence of taurine. Tomato tissue appeared to recover from the stress when treated with taurine after the induction of stress. Callus induction and shoot formation were significantly delayed compared to the stress-free controls. However, tomato tissue appeared to recuperate from the stress symptoms upon the treatment of taurine. FW increased to a greater extent in comparison to no-taurine control. The time for callus formation decreased after taurine treatment under the stress conditions. In addition, the reduced number of shoots in each stress condition could be restored by taurine. These observations strongly indicate that taurine may counteract the effects of stress caused by the two stress factors in a dose-dependent manner in tomato cells cultured in vitro. In conclusion, taurine can serve as an effective anti-stress agent in plant cells, as it does in animals.

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Part II
Taurine, Taurine Derivatives and Immune System

A Novel Cysteine Sulfinic Acid Decarboxylase Knock-Out Mouse: Immune Function

Seung Yong Park, Georgia Schuller-Levis, and Eunkyue Park

Abbreviations

CSAD	Cysteine sulfinic acid decarboxylase
CSAD KO	Cysteine sulfinic acid decarboxylase knockout mice
G1	G2 and G3, Generation 1, 2 and 3
G1 HO	HO ($CSAD^{-/-}$) mice born from HT ($CSAD^{+/-}$) parents
G2 or G3 HO	Mice born from G1 HO or G2 HO parents
HO	Homozygotic mice ($CSAD^{-/-}$)
HT	Heterozygotic mice ($CSAD^{+/-}$)
Tau T	Taurine transporter
WT	Wild type ($CSAD^{+/+}$)

1 Introduction

The effect of taurine on immune function has extensively been studied in taurine deficient cats as well as taurine-supplemented rodents and hamsters (Schuller-Levis and Park 2006; Sturman 1993; Gordon et al. 1992). Our data show that taurine deficient cats have a significant leukopenia, a shift in the percentage of polymorphonuclear leukocytes and mononuclear leukocytes, an increase in the leukocyte count, and a change in the sedimentation characteristics of leukocytes (Schuller-Levis

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et al. 1990; Schuller-Levis and Sturman 1992). Functional studies demonstrate a significant decrease in the respiratory burst and a decrease in phagocytosis. In addition, serum gamma globulin was significantly increased in taurine-deficient cats as well as histologic changes in lymph nodes and the spleen. Several in vivo models of oxidant-induced damage in rodents and hamster have been studied with supplementation of taurine to protect against subsequent inflammation. Hamsters pretreated with supplemental taurine and exposed to nitrogen dioxide did not show typical pathology associated with nitrogen dioxide damage (Gordon et al. 1992). Similarly, taurine and/or niacin reduced the inflammation and fibrosis resulting from bleomycin treatment in an animal model (Schuller-Levis et al. 2009; Wang et al. 1991). Ozone-induced lung inflammation was decreased by pretreatment of rodents with 5 % taurine in their drinking water for 10 days prior to ozone exposure (Schuller-Levis et al. 1995; Gordon et al. 1998). The number of inflammatory cells and hydroxyproline, markers for inflammation and fibrosis, were significantly reduced in taurine treated rats compared to untreated rats exposed to ozone. Thus, the maintenance of tissue taurine levels was critical to the prevention of oxidant-induced injury in several animal models.

Leukocytes capable of generating hypochlorous acid (HOCI) for hydrogen peroxide and chloride via the myeloperoxidase pathway have intracellular concentrations of taurine of 20–50 mM (Fudaka et al. 1982; Schuller-Levis and Park 2003, 2006)). Taurine reacts with HOCl to produce the less reactive and long-lived oxidant taurine chloramine (Tau-Cl) (Cantin 1994). Tau-Cl, a stable oxidant, downregulates the production of proinflammatory cytokines leading to a significant reduction in the immune response (Park et al. 1993, 1995; Marcinkiewicz et al. 1995; Quinn et al. 1996). Specifically, Tau-Cl inhibits proinflammatory mediators such as nitric oxide, tumor necrosis factor alpha, and prostaglandin E_2 by Tau-Cl in activated rodent cells. Tau-Cl suppressed superoxide anion, interleukin-6 (IL-6), and interleukin-8 (IL-8) production in activated human polymorphonuclear leukocytes (Park et al. 1998). The production of IL-6, IL-1beta, and IL-8 was also decreased in lipopolysaccharide activated adherent monocytes by Tau-Cl (Park et al. 2002). These data demonstrate the ability of Tau-Cl to modulate the immune response which is not species specific and extends to human leukocytes. Tau-Cl has also been shown to reduce IL-6 and IL-8 produced by fibroblasts-like synoviocytes isolated from patients with rheumatoid arthritis (Chorazy-Massalska et al. 2004; Kontny et al. 2006). Tau-Cl diminished the activity of NF-kB and to a lesser extent that of AP-1 transcription factor (Barua et al. 2002). Overall, the presence of high concentrations of taurine in leukocytes and the ability to form Tau-Cl in the presence of neutrophils coupled with effects on regulating non-adherent and adherent human leukocytes suggest a central role for taurine and its chloramine in regulating the immune response.

Recently we developed taurine deficient cysteine sulfinic acid decarboxylase knockout (CSAD KO) to further investigate the effect of taurine on immune function with and without taurine supplementation (Park et al. 2014). Alteration of immune function was found in both B and T cells from CSAD KO females and in B cells from CSAD KO males although taurine concentrations in both of females and males were not different significantly.

2 Materials and Methods

2.1 Reagents

LPS (Escherichia coli 0111:B4) was purchased from BD Biosciences (Sparks, MD). RPMI 1640, penicillin and streptomycin were obtained from Invitrogen (Carlsbad, CA). Heat inactivated fetal calf serum (FCS) was purchased from Gemini Products (Woodland, CA). Acetonitril, HPLC water, phenylisothiocyanate (PITC), triethylamine, Con A and taurine were obtained from Sigma Chemical (St. Louis, MO). ^{3}H-thymidine was obtained from American Radiolabeled Chemicals, Inc, St. Louis, MO.

2.2 CSAD KO Mice

CSAD KO mice were produced previously described from our laboratory (Park et al. 2014). Briefly, chimeric CSAD KO mice were produced by injection of cells from a gene trap ES cell line (XP0392) into C57BL/6 (B6) blastocysts at the Mouse Mutant Regional Resource Centers (MMRRC, UC Davis, CA) which were implanted into a pseudopregnant B6 mouse. Chimeric mice from MMRRC were mated with B6 (Jackson Laboratories, Bar Harbor, ME) in the animal colony at this institution (NYS Institute for Basic Research in Developmental Disabilities). Heterozygous siblings were mated to produce CSAD$^{-/-}$ homozygous pups (HO). Experimental mice were fed taurine-free chow (LabDiet®, PMI Nutrition International, St. Louis, MO). Taurine concentrations in commercial food were confirmed by HPLC. All mice were kept under 12-h day/night with free access to food and water. For optimum reproductive performance, one or two females were mated to a single male. Animals were weaned at 3–4 weeks of age. Generation 3 CSAD$^{-/-}$ (G3 CSAD$^{-/-}$, G3 HO) used in this study were born from G2 HO. G1 HO were born from heterozygotes (HT, CSAD$^{+/-}$). All procedures involving live animals were approved by the Institutional Animal Care and Use Committee of IBR.

2.3 Preparation of Splenocytes

After the spleens were removed from wild type (WT, CSAD$^{+/+}$) and third generation of homozygotes (G3 HO, CSAD$^{-/-}$), spleen and body weight were measured. A spleen index was calculated by (spleen weight/body weight) $\times$ 1,000. Splenocytes were prepared after lysis of red blood cells using lysis buffer (tris-buffered ammonium chloride). Cells suspended in complete media containing RPMI 1640 plus 10 % FCS and penicillin/streptomycin were counted by two independent researchers. After splenocytes (5×10^{5}/well) were added to a 96-well plate, LPS (1 μg/ml) or Con A (2.5 μg/ml) were added for mitogenic stimulation for B and T lymphocytes, respectively.

2.4 Lymphocyte Proliferation

Splenocytes (2×10^5 cells /well) were activated with Con A (2.5 μg/ml) or LPS (1.0 μg/ml) for 72 and 48 h at 37 °C in a 96 well plate, respectively (Cho et al. 2013). One microCurie of H^3-thymidine was added to each well 4 h before collection. H^3-thymidine incorporation was measured using a liquid scintillation counter (Packard Instrument Co., Meriden, CT).

2.5 High Performance Liquid Chromatography (HPLC)

The splenocytes (2×10^7 cells) were obtained from the spleen of WT and G3 HO at age of 8–12 weeks after lysis of red blood cells with tris-buffer ammonium chloride and kept in −70° C until used. Taurine concentrations were determined using HPLC (Waters, Milford, MA) (Battaglia et al. 1999). Briefly, frozen splenocytes were melted twice and were homogenized using 5 % TCA and centrifuged for removal of proteins. Samples were dried using a Speedvac (Savant, Holbrook, NY) and derivatized using phenylisothiocyanate (PITC), and separated using a C18 column with a gradient of acetate buffer containing 2.5 % acetonitril (pH 6.5) and 45 % acetonitril solution containing 15 % methanol at 45 °C. The flow rate was 1 ml/min. Taurine concentrations were determined by comparison to a standard.

2.6 Statistical Analysis

Differences between control and experimental groups were determined by Student's independent *t*-test as well as LSD and Tukey analysis under breakdown and ANOVA using Statistica (Statsoft, Tulsa, OK). Statistical significance of differences between the groups is considered at the level of $p < 0.05$.

3 Results

3.1 Comparison of Body Weight, Spleen Index and Splenocyte Taurine Concentrations in WT and G3 HO

Body weight and spleen index in WT and G3 HO were not different significantly. In addition, there was no significant difference in body weight and spleen index in female and males in WT and G3 HO (Table 1). Taurine concentrations in G3 HO were significantly decreased by 56 % compared to WT. Taurine concentrations of females and males in WT and G3 HO were not different.

Table 1 Body weight, spleen index and splenocyte taurine concentrations in CSAD KO

Sex	GT	Body weight	Spleen index	Tau in splenocytes[a]
Female[b]	WT	21.5 ± 0.9[c]	3.3 ± 0.3	18.1 ± 1.0
	G3 HO	20.6 ± 0.5	2.9 ± 0.2	10.2 ± 0.5*
Male[b]	WT	25.5 ± 0.7	3.9 ± 0.4	18.7 ± 0.8
	G3 HO	25.8 ± 0.7	3.7 ± 0.2	12.2 ± 0.7*

*Significantly different compared to WT, $p < 0.0002$

[a]The unit of taurine concentrations is nmol/10^7 splenocytes

[b]The number of animals in each group of female and male is 8 and 9, respectively

[c]Data represent mean ± SE (g)

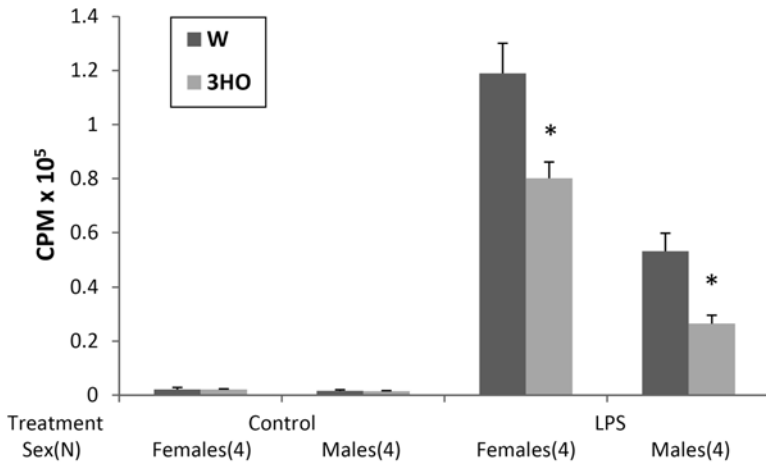

Fig. 1 Lymphocyte proliferation of splenocytes with LPS (1 μg/ml) in females and males at age of 8–12 weeks. Number of mice per group is four each in controls and LPS-activated groups. One additional experiment demonstrated similar results. *Asterisk* is significantly different compared to WT ($p < 0.05$)

3.2 *Lymphocyte Proliferation with LPS*

In order to compare B lymphocyte proliferation between WT and G3 HO, LPS-induced lymphocyte proliferation was measured using ^{3}H-thymidine incorporation for DNA synthesis. Cellular proliferation in both females and males from G3 HO was significantly decreased by 30 % compared to WT (Fig. 1). The effect of exogenous taurine on lymphocyte transformation was examined using 10 and 50 mM of taurine simultaneously treated with LPS. Both 10 and 50 mM of taurine concentrations were without effect (Table 2).

Table 2 Effect of exogenous taurine on lymphocyte proliferation with LPS

Sex	GT	LPS	LPS + taurine 10[a]	LPS + taurine 50[a]
Female	WT	118,989 ± 11,150[b]	109,084 ± 17294	111,163 ± 21,726
	G3 HO	80,098 ± 6,034*	75,172 ± 5,798	75,969 ± 7,872
Male	WT	53,325 ± 6,464	47,620 ± 6,890	55,256 ± 10,669
	G3 HO	26,507 ± 3,113*	24,490 ± 2,408	22,716 ± 2,701

*Significantly different compared to WT, $p < 0.05$

[a]Two concentrations of taurine including 10 and 50 mM were used. The LPS concentration was 1 μg/ml

[b]Data represent mean ± SE (CPM). Four mice were used in all groups. One additional experiment showed similar results

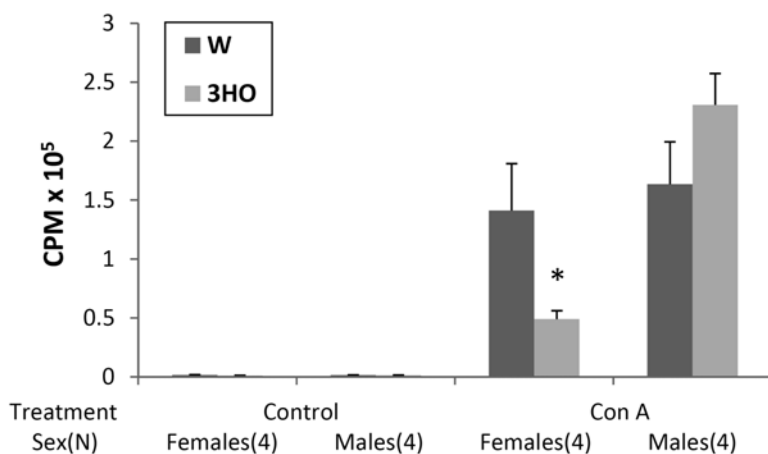

Fig. 2 Lymphocyte proliferation of splenocytes with Con A (2.5 μg/ml) in females and males at age of 8–12 weeks. Number of mice per group is four each in controls and Con A-activated groups. One additional experiment demonstrated similar results. *Asterisk* is significantly different compared to WT ($p < 0.05$)

3.3 Lymphocyte Proliferation with Con A

T lymphocyte proliferation was measured using Con A, a T lymphocyte mitogen. T lymphocyte proliferation in females from G3 HO was significantly decreased by 30 % compared to females from WT (Fig. 2). However, cellular proliferation in males from G3 HO was not increased significantly compared to males from WT. In order to examine whether exogenous taurine affects lymphocyte transformation, two taurine concentrations including 10 and 50 mM were added to splenocytes treated simultaneously with Con A. Both concentrations of taurine were without effect (Table 3).

Table 3 Effect of exogenous taurine on lymphocyte proliferation with Con A

Sex	GT	Con A	Con A + taurine 10[a]	Con A + taurine 50[a]
Female	WT G3 HO	141,132 ± 39,841[b] 49,056 ± 7,125*	134,019 ± 35,866 45,963 ± 5,414	125,469 ± 29,123 40,752 ± 3,278
Male	WT G3 HO	163,477 ± 35,980 230,712 ± 26,677	166,947 ± 46,462 229,771 ± 17,640	160,807 ± 35,209 233,059 ± 21,930

*Significantly different compared to WT, $p < 0.05$

[a]Two concentrations of taurine (10 and 50 mM) were used. The concentration of Con A was 2.5 μg/ml

[b]Data represent mean ± SE (CPM). Four mice were used in all groups. One additional experiment showed similar results

4 Discussion

A novel taurine deficient CSAD KO was developed in our laboratory (Park et al. 2014). Taurine supplementation in this model prior to and during pregnancy rescues neonatal mortality. Some genes including antioxidant enzymes and metabolic enzymes were restored using taurine supplementation. Since taurine deficiency in cats shows severe abnormalities in immune function, females and males from CSAD KO mice were examined for altered immune function including lymphocyte proliferation, spleen index and determination of taurine concentrations (Sturman 1993; Schuller-Levis and Park 2003). T and B lymphocytes, the major cells in adaptive immunity, the secondary defense system, and polymorphonuclear leukocytes (PMN) and macrophages, the cells of the primary innate immunity were examined (Schuller-Levis et al. 1990; Schuller-Levis and Sturman 1992). Recently innate lymphoid cells such as natural killer cells and lymphoid cell group 1, 2 and 3 which lack T cell receptor complexes and immunoglobulin receptors are extensively studied for the defense of microorganisms (Diefenbach 2013; Spits and Cupedo 2012; Tanriver and Defenbach 2014). These leukocytes remove foreign substances such as oxidants and microorganisms through innate and adaptive immune systems. In this study, our data demonstrated that adaptive immune function, including B and T lymphocytes, was significantly reduced in taurine-deficient CSAD KO compared to WT although the spleen index was unaffected compared to WT (Table 1). LPS and Con A activated lymphocyte proliferation was significantly decreased (Figs. 1 and 2), especially in females. Con A-induced lymphocyte transformation in males differed from females. Although LPS-induced lymphocyte proliferation in males from G3 HO was lower than WT, Con A-induced lymphocyte proliferation was increased but without significance. Taurine concentrations in splenocytes from both females and males from G3 HO and WT were not different (Table 1). These data demonstrated that polyclonal lymphocyte activation maybe regulated by sex hormones. In order to examine whether exogenous taurine restores immune function in G3 HO, taurine was added to both LPS- and Con A-induced lymphocyte proliferation, but was without effect (Tables 2 and 3). These data indicate reduced immune function

which may be attributed to the lack of a CSAD gene instead of low taurine concentrations. These data also suggested that the CSAD gene may be involved in regulation of genes related to lymphocyte proliferation.

Taurine concentrations in the splenocytes transported through Tau T were significantly higher compared to those in the liver and plasma (Table 1) (Uchida et al. (1992), unpublished data), indicating taurine is required for critical antioxidant and/or anti-inflammatory function. Several laboratories demonstrated taurine protects against inflammatory injury in various organs under oxidative stress in animal models (Gordon et al. 1992, 1998; Wang et al. 1991; Schuller-Levis et al. 1995, 2009). This protection may be mediated by either taurine itself or its metabolite, Tau-Cl. Tau-Cl is produced by myeloperoxidase in the presence of halides such as hyphochlorite when PMNs are activated by invasion of foreign substances (Schuller-Levis and Park 2003; Cantin 1994). Previously, our laboratory demonstrated that taurine protects against oxidant-induced injury in rats and this protection may be mediated by Tau-Cl. Tau-Cl inhibits proinflammatory mediators including superoxide anion, nitric oxide, TNF-α, PGE ^{2}, IL-6 and IL-8 in activated macrophages and PMNs (Schuller-Levis and Park 2006; Park et al. 1993, 1995, 1998, 2002; Marcinkiewicz et al. 1995; Kim et al. 1996). Tau-Cl has also been shown to reduce IL-6 and IL-8 produced by fibroblast-like synoviocytes isolated from patients with rheumatoid arthritis, indicating that it may down-regulate rheumatoid arthritis (Chorazy-Massalska et al. 2004; Kontny et al. 2006). We demonstrated that Tau-Cl inhibits LPS- and Con A-induced human lymphocyte proliferation (Park et al. 2002). These data indicated that taurine and/or its metabolite, Tau-Cl, play a critical role in regulation of immune function and inflammatory responses importantly in both humans and animals. Since previous experiments for the role of taurine in protection of oxidative injury were performed using rats and mice with high taurine concentrations, taurine deficient CSAD KO provide a model to better define the role of taurine in immune function and the inflammatory response with or without taurine supplementation. Therefore, a novel taurine-deficient CSAD KO will be an indispensable animal model for further studies on the role of taurine and the CSAD gene in immune function and the inflammatory response.

5 Conclusion

Immune function in taurine-deficient CSAD KO was reduced significantly compared to WT. Taurine concentrations in both females and males from G3 HO were significantly lower than WT. However, the decrease of taurine concentrations in splenocytes was significantly less compared to those in the liver and plasma. Our data suggested that taurine and the CSAD gene is required for normal immune function and abnormal immune function may be corrected with taurine supplementation.

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Taurine Chloramine, a Taurine Metabolite from Activated Neutrophils, Inhibits Osteoclastogenesis by Suppressing NFATc1 Expression

Chaekyun Kim and In Soon Kang

Abbreviations

BMMs	Bone marrow-derived monocyte/macrophage precursor cells
CGD	Chronic granulomatous disease
CIA	Collagen-induced arthritis
IL	Interleukin
M-CSF	Macrophage colony-stimulating factor
MPO	Myeloperoxidase
NFATc1	Nuclear factor of activated T cell 1
NF-κB	Nuclear factor-κB
NO	Nitric oxide
RANKL	Receptor activator of nuclear factor-κB ligand
TauCl	Taurine chloramine
TNF	Tumor necrosis factor
TRAP	Tartrate-resistant acid phosphatase

1 Introduction

Taurine is one of the most abundant free amino acids and plays important roles in many biological processes (Learn et al. 1990; Huxtable 1992). It is stored in inflammatory cells at a concentration of up to 50 mM and has been reported to protect cells

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at sites of inflammation against the injury caused by overproduction of cytotoxic reactive oxygen species (ROS) during inflammation. The protection of inflammatory cells by taurine is primarily the result of its efficient elimination of highly toxic hypochlorite ($HOCl/OCl^-$) and the generation of less toxic taurine chloramine (TauCl) in activated neutrophils.

Taurine reacts with $HOCl/OCl^-$ produced by the halide-dependent myeloperoxidase (MPO) system and becomes TauCl. The requirement of superoxide anion (O_2^-) for TauCl generation has been well established in neutrophils, so that only O_2^- producing neutrophils, but not non-O_2^- producing cells nor the neutrophils of chronic granulomatous disease (CGD) patients, possess the ability to produce TauCl (Kim and Cha 2014). TauCl has been demonstrated to suppress the production of many inflammatory mediators, such as O_2^-, nitric oxide (NO), tumor necrosis factor (TNF)-α, interleukin (IL)-1β, -2, -6, -8, and -10, prostaglandin E_2, macrophage inflammatory protein-2, monocyte chemoattractant protein-1 and -2, and matrix metalloproteinase (Kim and Cha 2014; Marcinkiewicz and Kontny 2014). In addition, in macrophages, exposure to TauCl increases the level of many antioxidant proteins, such as heme oxygenase-1, glutathione peroxidase, peroxiredoxin and catalase, which scavenge and diminish the production of cytotoxic reactive oxygen metabolites (Sun Jang et al. 2009; Kim et al. 2010). Thus, the cytoprotective, anti-inflammatory and anti-oxidant effects of TauCl protect cells and tissues during inflammation and accelerate the resolution of inflammation.

Bone homeostasis is a complex but well orchestrated process that is mediated by dynamic balance between osteoblastic bone formation and osteoclastic bone resorption (Manolagas 2000). Osteoclasts originate from bone marrow-derived monocyte/macrophage precursor cells (BMMs), and their differentiation is regulated mainly by macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL). M-CSF promotes proliferation and survival of BMMs and RANKL activates osteoclastic differentiation process by inducing the master transcription factor for osteoclastogenesis, nuclear factor of activated T cell 1 (NFATc1) (Takayanagi et al. 2002).

In our previous study, we showed that TauCl significantly reduces synovial inflammation, cartilage damage and bone erosion by inhibiting lymphocyte proliferation and osteoclast formation in mice with collagen-induced arthritis (CIA) (Wang et al. 2011). In this study, we further investigated the effect of TauCl on osteoclast differentiation and the mechanism involved. TauCl was found to inhibit osteoclast differentiation from BMMs in a dose-dependent manner. In addition, TauCl inhibited the expression of osteoclast markers like tartrate-resistant acid phosphatase (TRAP) and cathepsin K, reduced TRAP activity, and inhibited the induction of osteoclast-specific transcription factor NFATc1.

2 Materials and Methods

2.1 Mice

C57BL/6J mice (Jackson) were housed under pathogen-free conditions at the animal facility in Inha University. All experiments were conducted in accordance with institutional guidelines approved by the Animal Care and Use Committee of Inha University (INHA 131217-255).

2.2 Synthesis of TauCl

TauCl was synthesized immediately before experiments by adding equimolar amounts of sodium hypochlorite (NaOCl) to taurine dissolved in 1.8 % NaCl (Kim et al. 2010). Reagents were purchased from Sigma-Aldrich, unless otherwise specified.

2.3 Osteoclast Differentiation

Bone marrow cells obtained from 6- to 8-week-old male C57BL/6J mice were cultured overnight in α-MEM (Hyclone) containing 10 % FBS and 10 ng/ml M-CSF (Peprotech). Nonadherent cells were further differentiated to preosteoclasts in α-MEM containing 30 ng/ml M-CSF and 10 % FBS for 3 days. Preosteoclasts were then transferred to 48-well plates and cultured for 4 more days in α-MEM containing 10 % FBS, 30 ng/ml M-CSF and 50 ng/ml RANKL (Peprotech) in the presence of TauCl (0.02, 0.05, 0.1, 0.2 and 0.5 mM). RANKL-driven osteoclastogenesis was assessed by counting TRAP-positive cells containing more than three nuclei.

2.4 TRAP Staining

The presence of TRAP (acid phosphatase 5) activity is regarded as an important cytochemical marker of osteoclastogenesis. Cells were fixed with 3.7 % formaldehyde for 5 min and incubated with 0.1 % Triton X-100 for 5 min, and then stained using the Leukocyte Acid Phosphatase Assay kit. In brief, cells were incubated in a mixture of solutions containing Fast Garnet GBC base, sodium nitrite, naphthol AS-BI phosphoric acid, acetate, and tartrate for 1 h at 37 °C in the dark, and then

washed with water. Cells were then counterstained with hematoxylin solution, Gill No. 3, for 2 min at room temperature. TRAP-positive multinucleated cells were counted as osteoclasts.

2.5 TRAP Activity

Cells were incubated in 50 mM citrate buffer (pH 4.5) containing 10 mM sodium tartrate and 5 mM 4-nitrophenylphosphate for 30 min. Mixtures were then transferred to new plates containing an equal volume of 0.1 N NaOH. Absorbance was measured at 405 nm using Versamax microplate reader (Molecular Devices) equipped with SOFTMAX software.

2.6 Quantitative Real Time PCR

Total RNA was extracted using TRI reagent (MRC) according to the manufacturer's instructions. Reverse transcription of 200 ng of total RNA was performed according to the instructions provided by Takara. PCR amplification of mRNA was carried out on an Applied Biosystems StepOne unit using SYBR Green PCR Master Mix and the following primers (forward and reverse, respectively), TRAP, 5′-ACG GCT ACT TGC GGT TTC A-3′ and 5′-TCC TTG GGA GGC TGG TCT T-3′; cathepsin K, 5′-GAA GAA GAC TCA CCA GAA GCA G-3′ and 5′-TCC AGG TTA TGG GCA GAG ATT-3′; GAPDH, 5′-CCT TCC GTC CTA CCC C-3′ and 5′-CCC AAG ATG CCC TTC ATG-3′. The amplification data were analyzed using the sequence detection software from Applied Biosystems.

2.7 Western Blot Analysis

Cell lysates were prepared from osteoclasts as described previously (Kim and Dinauer 2001) and 20–30 μg of total proteins were electrophoresed on SDS-PAGE. Resolved proteins were transferred onto polyvinylidene fluoride membranes (BioRad), probed with specific antibodies against NFATc1 (Santa Cruz) and β-actin, and developed using the ECL method (Amersham).

2.8 Statistical Analysis

Two-tailed Student's t-test (paired) was performed using Microsoft Excel software. Results were expressed as mean ± SD, and p values < 0.05 were considered statistically significant.

3 Results

3.1 TauCl Inhibited Osteoclast Differentiation

Osteoclasts are formed from well differentiated BMMs in response to RANKL in the presence of M-CSF. RANKL is expressed on osteoblasts and induces the signaling essential for osteoclastogenesis (Yasuda et al. 1998; Theill et al. 2002), and M-CSF is secreted by osteoblasts and provides the survival signal (Yoshida et al. 1990). The effect of TauCl on osteoclast differentiation was evaluated by counting numbers of the multinucleated TRAP-positive cells. RANKL-induced increases in osteoclast numbers were dose-dependently inhibited by TauCl (Fig. 1), and completely prevented by 0.2 mM TauCl (data not shown). At the concentrations used TauCl had no effect on cell viability.

3.2 TauCl Inhibited mRNA Expression of Osteoclast-Specific Markers and TRAP Activity

TRAP and cathepsin K serve as histochemical markers of osteoclasts, in which they are highly expressed. In addition, TRAP plays critical roles in collagen synthesis and degradation, bone mineralization, skeletal development, cytokine production and Th1 responses. On the other hand, cathepsin K is a lysosomal cysteine proteinase that is abundant in osteoclasts and plays a role in bone resorption and remodeling.

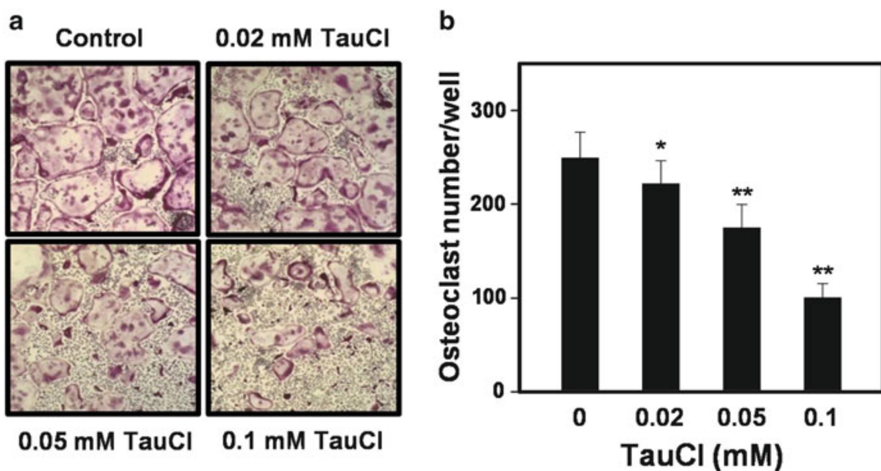

Fig. 1 Effect of TauCl on RANKL-induced osteoclasts generation. (**a**) Osteoclast differentiation from BMMs in response to RANKL and M-CSF (×100). (**b**) Numbers of multinuclear (nuclei ≥3) TRAP-positive cells. Results are presented as mean ± SD (n = 3), *$p < 0.05$ and **$p < 0.01$

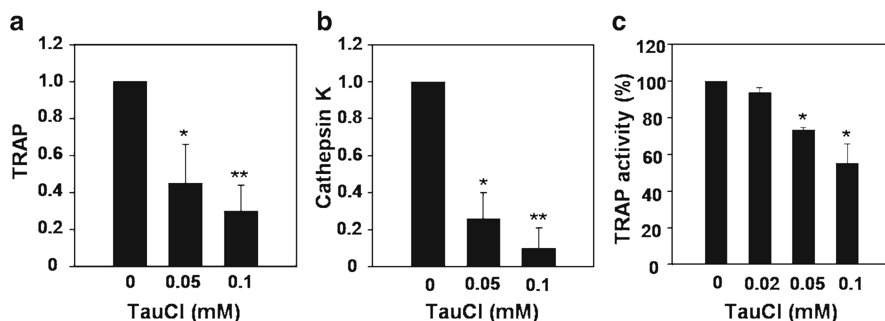

Fig. 2 Effect of TauCl on the expression and activity of osteoclast-specific markers. (**a**, **b**) The mRNA expressions of TRAP and cathepsin K in osteoclasts. (**c**) The effect of TauCl on TRAP activity. Results are presented as mean ± SD (n = 3), *p < 0.05 and **p < 0.01

Here, we investigated the effect of TauCl on mRNA expressions of genes associated with osteoclast-specific markers. TauCl inhibited the expression of TRAP and cathepsin K in osteoclasts in a dose-dependent manner (Fig. 2a, b). We also investigated the effect of TauCl on TRAP activity. TauCl decreased the number of TRAP-positive cells and TRAP activity (Figs. 1a and 2c).

3.3 *TauCl Inhibited RANKL-Stimulated NFATc1 Expression*

A number of transcription factors, such as PU.1, c-Fos, NF-κB and NFATc1 play critical roles during osteoclastogenesis (Grigoriadis et al. 1994; Teitelbaum 2000), and NFATc1 is the transcription factor most strongly induced by RANKL stimulation (Takayanagi et al. 2002). NFATc1 regulates a number of osteoclast-specific genes, such as, TRAP, cathepsin K, and beta3 integrin. Stimulation with 50 ng/ml RANKL induced NFATc1 protein expression in osteoclasts, and that RANKL-induced NFATc1 expression was inhibited by TauCl (Fig. 3). However, TauCl did not affect the expression of NF-κB, although it did tend to inhibit the phosphorylation of NF-κB (data not shown). These observations suggest that NFATc1 plays a crucial role in TauCl-induced osteoclastogenesis inhibition.

4 Discussion

The present study was undertaken to investigate the effect of TauCl on osteoclast differentiation and to determine the mechanism involved. It was found that TauCl inhibited osteoclast differentiation from BMMs in a dose-dependent manner. In addition, TauCl also inhibited the expression of osteoclast markers like TRAP and cathepsin K, reduced TRAP activity, and inhibited the induction of osteoclast-specific transcription factor NFATc1.

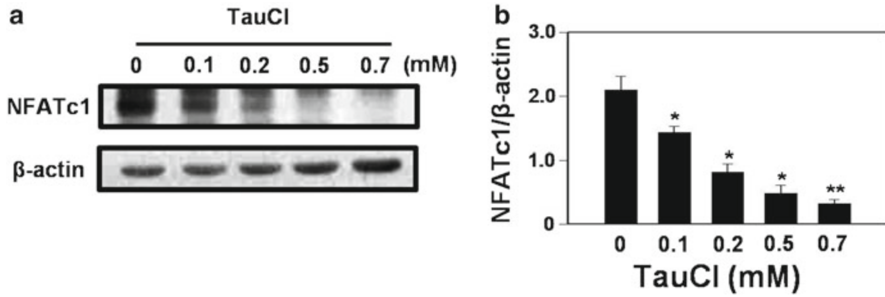

Fig. 3 Effect of TauCl on RANKL-induced NFATc1 expression. (**a**) Expression of NFATc1 in osteoclasts after 96 h of RANKL stimulation. (**b**) *Bar graph* showing levels of NFATc1, as determined by densitometry of immunoblot signals and normalized to β-actin. Results are presented as mean ± SD (n = 3), *p < 0.05 and **p < 0.01

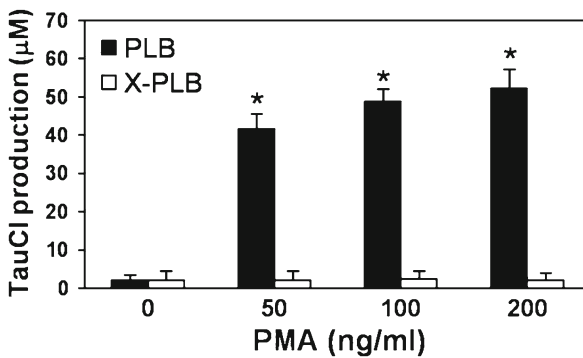

Fig. 4 The production of TauCl in neutrophils requires effective NADPH oxidase activity. Human myeloid cell lines, PLB-985 and X-PLB cells (2×10^6) were stimulated with PMA for 30 min, and TauCl production was then determined by quantifying the oxidation of 3,3′,5,5′-tetramethylbenzidine at 650 nm. Results are presented as mean ± SD (n = 3), *p < 0.01

When inflammation occurs, neutrophils infiltrate into infected tissues and play a prominent role as the first-line defense by phagocytosing invading microbes and killing them by releasing ROS and granules. TauCl is produced in activated neutrophils by a reaction between taurine and $HOCl/OCl^-$, and then released into the inflammatory milieu. TauCl production required the O_2^- derived from phagocytic NADPH oxidase. Only O_2^- producing neutrophil-like PLB-985 cells, but not X-PLB (NADPH oxidase gp91*phox*-null) cells, produced TauCl (Fig. 4). Thus, most TauCl is produced from the O_2^- generated as a result of the oxidative burst catalyzed by NADPH oxidase in activated neutrophils in an inflammatory environment.

Our team and other authors have shown that TauCl significantly reduces synovial inflammation, cartilage damage, and bone destruction in mice with CIA (Kwasny-Krochin et al. 2002; Wang et al. 2011). Furthermore, the enhancement of bone resorption is known to be closely associated with inflammatory processes that drive

excess osteoclast formation. In a previous study, we found TauCl inhibited osteoclast formation in the bones of CIA mice and in RANKL-induced BMMs, and these observations were confirmed during the present study (Wang et al. 2011; Fig. 1). In addition, TauCl inhibited the expression of osteoclast-specific genes like TRAP, cathepsin K, and NFATc1. The latter of which regulates a number of cytokine genes in immune cells (Robbs et al. 2008) and controls osteoclast-specific genes in osteoclasts. TauCl inhibited the production of inflammatory cytokines, such as TNF-α, IL-1 and IL-6, which are important for bone destruction (Marcinkiewicz and Kontny 2014 ; Kim and Cha 2014). Taken together, these results suggest that TauCl reduces inflammation-derived bone destruction by inhibiting osteoclast formation.

5 Conclusion

In summary, TauCl was found to inhibit RANKL-induced osteoclast differentiation, the expressions of osteoclast markers, such as TRAP and cathepsin K, and TRAP activity. Furthermore, TauCl inhibited the expression of NFATc1 (a key transcription factor required for osteoclastogenesis), which regulates the expression of TRAP and cathepsin K. Thus, TauCl is produced endogenously by a phagocytic oxidative burst from activated neutrophils and inhibits RANKL-induced osteoclast differentiation by inhibiting the expression of NFATc1.

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Impact of Taurine on Innate and Adaptive Immunity as the Result of HOCl Neutralization

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Abbreviations

A1AT	Alpha1-antitrypsin
BAPNA	a-*N*-benzoyl-DL-arginine-*p*-nitroanilide
BSA	Bovine serum albumin
HOCl	Hypochlorous acid
LPS	Lipopolysaccharide
MPO	Myeloperoxidase
MSA	Mouse native albumin
MSA-Cl	Mouse chlorinated albumin
NE	Neutrophil elastase
OVA	Native ovalbumin
OVA-Cl	Chlorinated ovalbumin
ROS	Reactive oxygen species
SOD	Superoxide dismutase
Tau	Taurine
TauCl	Taurine chloramines (*N*-chlorotaurine)

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1 Introduction

Taurine (2-aminoethanesulfonic acid) is the most abundant free amino acid in mammalian cells (Learn et al. 1990). It is commonly accepted that taurine plays an important role as an antioxidant to protect cells from oxidative stress (Schaffer et al. 2009; Oliveira et al. 2010; Wang et al. 2009). Therefore, the primary role of taurine seems to be cytoprotection and maintaining homeostasis of cells involved in acute and chronic inflammation, the reactions associated with the generation of reactive oxygen species (ROS) (Huxtable 1992; Klebanoff 2005; Schuller-Levis and Park 2004). Much less is known about the impact of taurine on neutrophil functions in innate and adaptive immunity. However, exceptionally high concentration of taurine in neutrophil cytosol indicates its special role in biology of these cells (Weiss et al. 1982; Wang et al. 2009).

Neutrophils are essential effector cells in acute infection being the first line defense cells of innate immunity (Klebanoff 1968; Smith 1994). Neutrophils kill pathogens through generation of microbicidal proteases and ROS (Fig. 1) (Thomas 1979; Babior 1978). It has also been suggested that neutrophils may provide a link between innate and adaptive immunity (Marcinkiewicz 1997).

However, it is difficult to establish whether neutrophils influence T cell or B cell responses during infection (Marcinkiewicz et al. 1998, 1999). On the other hand, there is a growing number of data indicating that oxidative modification of proteins with hypochlorous acid (HOCl) may alter their biological functions, including their immunogenicity (Marcinkiewicz et al. 1991, 1992; Prokopowicz et al. 2010).

HOCl, the major product of the neutrophil MPO-halide system is a key molecule of acute inflammation and innate immunity (Thomas 1979). HOCl is involved in bacterial killing inside phagolysosomes (Klebanoff 1968). HOCl is an extremely reactive oxidant and may react with a variety of substances at a site of inflammation including proteins as the primary targets (Weiss 1988; Hawkins and Davies 1999). Oxidative modification of proteins with HOCl is characterized by chlorination of amino groups in proteins, either N-terminal, or on the side chains of histidine, lysine and arginine to form mono- and di-chloroamines (Hawkins and Davies 1999; Pattison and Davies 2001). These chloramines show weaker oxidant activity, but are much longer-lived than HOCl itself or free amino acid chloramines (Hawkins and Davies 1999). Chloramines formed in these reactions are subsequently converted into free aldehydes and irreversibly oxidized to carboxylic acids (Allison and Fearon 2000). However, the primary chloramine generated at a site of inflammation is taurine chloramine (TauCl), mild oxidant with anti-inflammatory and anti-microbial activity (Park et al. 1995, 2000; Marcinkiewicz et al. 1995; Kim and Cha 2009; Nagl et al. 2000). In addition, tryptophan, tyrosine, cysteine and methionine residues are well known targets for chlorination. Importantly, it has been shown that HOCl may alter reactive center of a number of proteins containing target amino acids, especially methionine (Peskin and Winterbourn 2001). Importantly, methionine oxidation either activates (Claesson et al. 1996) or inactivates (Davies et al. 1994) enzymes involved in inflammation. Moreover, it has been documented that *in vitro*

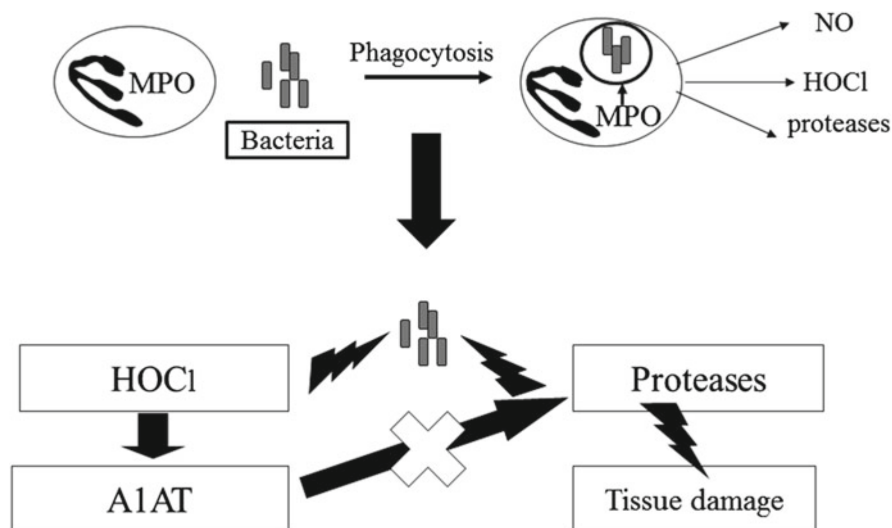

Fig. 1 Microbicidal agents of neutrophils. Enhancement of protease activity by HOCl *via* inactivation of proteinase inhibitor (alfa1-antitrypsin)

modification of protein antigens with HOCl enhances their immunogenicity (Marcinkiewicz et al. 1991). HOCl has a positive role in the CD4 T cell and B cell responses to variety of proteins, such as hen egg lysozyme, bovine alpha-lactoalbumin, bovine gammaglobulin and ovalbumin (OVA), the best studied model of protein chlorination (Marcinkiewicz et al. 1992; Allison and Fearon 2000; Prokopowicz et al. 2010).

In the present study we have tested the impact of taurine on HOCl-mediated protein oxidation. First, we have compared the effect of HOCl with that of TauCl, the product of HOCl reaction with taurine on activity of alpha1-antitrypsin (A1AT), the major inhibitor of neutrophil serine proteases and a potent anti-inflammatory agent . Secondly, we have asked the question whether HOCl-oxidative modification of self (mouse albumin) and non-self (ovalbumin) proteins can effectively enhance their antigenicity/immunogenicity, when performed in the presence of taurine.

2 Methods

2.1 Chemicals

N-Chlorotaurine, a kind gift from Prof. Waldemar Gottardi and Prof. Marcus Nagl from the Division of Hygiene and Medical Microbiology, Innsbruck Medical University, Austria, Taurine, HOCl, OVA, BSA, BAPNA (Sigma Aldrich), mouse albumin fraction V (MSA) (ICN biomedicals Inc), L-methionine (Fluka), thiosulphate (BDH Chemicals).

2.2 Preparation of TauCl

TauCl (*N*-chlorotaurine sodium salt) as a crystalline sodium salt (molecular weight 181.57) was prepared as described previously (Gottardi and Nagl 2002). Each preparation of TauCl was monitored by UV absorption spectra ($\lambda = 200–400$ nm) to assure the authenticity of TauCl ($\lambda = 252$ nm) and the absence of dichloramine ($TauCl_2$) ($\lambda = 300$ nm) and unreacted HOCl/OCl ($\lambda = 292$ nm). The concentration of synthesized TauCl was determined using the molar extinction coefficient factor $\varepsilon = 429\ M^{-1}\ cm^{-1}$ at A_{252}.

2.3 Animals

Inbred CBA/J mice (8–12 weeks of age, 18–22 g) were maintained in the Animal Breeding Unit, Department of Immunology, Jagiellonian University Medical College, Cracow. All mice were housed in the laboratory room with water and standard diet *ad libitum*. The authors were granted permission by the Local Ethical Committee to use mice in this study. Each experimental group consisted of six animals.

2.4 Measurement of A1AT Activity

To determine the effect of Tau, HOCl and TauCl on A1AT we used the modified enzymatic assay of alpha1-antitrypsin activity (Dietz et al. 1974). This method is based on the ability of A1AT to inhibit the hydrolysis of a-*N*-benzoyl-DL-ARGININE-P-nitroanilide (BAPNA) by trypsin to *p*-nitroaniline (PNA) and benzoili-(D,L)-arginine. The absorbance of PNA, measured at 405 nm is proportional to the trypsin activity.

Briefly, A1AT was incubated with different concentrations of the tested substances for 15 min at 37 °C. As a control sample (positive control) A1AT solution was mixed with the reaction buffer (Tris pH 8.48–100 mM, 20 mM $CaCl_2$). After the incubation, all samples were diluted 100× in the reaction buffer and mixed with 1 mM BAPNA. Than trypsin solution (40 mg/ml) was added and plate was incubated for 10 min at 37 °C. After incubation the enzymatic reaction was stopped by adding 30 ml of acetic acid (30 %) and absorbance was measured at 405 nm. The influence of tested substances on activity of A1AT is showed as a percent of activity of a positive control.

2.5 Protein Chlorination

Chlorination, oxidative modification of proteins was performed as described before (Prokopowicz et al. 2010). Proteins (OVA, MSA) (2 mg/ml) were incubated with 1 mM of HOCl solution in the presence or absence of taurine (30 mM). The HOCl

concentration was determined before each reaction by UV absorption spectra measurement at 292 nm using extinction coefficient factor $\varepsilon = 350\ M^{-1}\ cm^{-1}$. The reaction was incubated at 37 °C for 1 h. HOCl excess was removed by L-methionine at concentration ratio 0.44 mg of methionine per 1 mg OVA/MSA. Then, the excess of methionine was dialyzed off against outer 0.005 phosphate buffer pH 7.4 for 24 h at 4 °C. The ratio of external to internal compartment volume was 100/1.

2.6 Mice Immunization

2.6.1 OVA

Mice were immunized with: native OVA, chlorinated OVA (OVA-Cl) and OVA chlorinated in the presence of taurine (OVA-Cl-Tau) 200 μg/protein per mouse and all mice received 1 μg of LPS as an adjuvant by intraperitoneal (i.p). injection (primary immunization). On day 14, mice were rechallenged with 100 μg OVA and 1 μg LPS via i.p. injection (boost). Five days later, mice were sacrificed and level of IgG, IgG1 and IgG2a anti-native OVA was estimated.

2.6.2 MSA

Mice were immunized with: native MSA, chlorinated MSA (MSA-Cl) and MSA chlorinated in the presence of taurine (MSA-Cl-Tau) 200 μg/protein per mouse and all mice received 1 μg of LPS as an adjuvant by intraperitoneal (i.p). injection (primary immunization). On day 14, mice were rechallenged with 100 μg MSA and 1 μg LPS via i.p. injection (boost). Five days later, mice were sacrificed and level of IgG anti-MSA-Cl was estimated.

2.7 Measurement of Serum OVA-Specific or MSA-Cl Specific Antibody Titer

The level of antibodies specific to OVA, MSA-Cl in mouse serum was measured by ELISA. Briefly, plates were coated overnight with OVA or MSA-Cl (5 μg/ml). Serial dilutions of mouse serum in PBS were applied to 96-well antigen-coated plates (Costar, USA) for 1 h at room temperature. Immunoglobulins in tested serum samples were detected using biotin-conjugated antibodies against IgG (1:10,000, Sigma-Aldrich, Germany), IgG1 (1:10,000, MP Biomedicals, USA), IgG2a (Sothern Biotech, USA). The ELISA was developed using horseradish peroxidase conjugated with streptavidin (Vector, USA) followed with o-phenylenediamine and H_2O_2 (both Sigma-Aldrich, Germany). After 30 min reaction was stopped by addition of 3 M H_2SO_4. The optical density of each sample was measured at 492 nm in

Power Wave microplate reader (Bio-Tek Instruments, USA). 0.05 % Tween-20 in phosphate buffer was used as washing solution. The amount of antibody was expressed in arbitrary units: 1 U = 1/100 titers of antibodies specific to OVA, and MSA-Cl.

2.8 Statistics

Statistical significance between two groups was tested using Student's t test. For more groups, one-way ANOVA comparison and Tukey post hoc test were used. Results are expressed as a mean ± SEM values. A p value <0.05 was considered statistically significant. Analysis was performed using GraphPad Prism ver. 5.01 program (GraphPad Software, USA).

3 Results and Discussion

3.1 Innate Immunity: Taurine Ameliorates HOCl-Related Pro-inflammatory Activity—Effects of HOCl, Tau and TauCl on Enzymatic Activity of Alpha1-Antitrypsin

Neutrophils, the primary cells of innate immunity and inflammation are specialized for killing microorganisms (Klebanoff 1968). They are also involved in a pathogenesis of inflammatory diseases and responsible for tissue injury (Weiss 1988; Smith 1994). Among a number of antimicrobial systems of phagocytes, the MPO-H2O2-chloride system and its major product, HOCl, are specific for neutrophils (Thomas 1979). HOCl, strong oxidant with well documented microbicidal activity, reacts readily with a variety of biological targets including proteins (Weiss et al. 1982; Klebanoff 2005; Marcinkiewicz et al. 2000). HOCl-mediated oxidative modification (chlorination) of self-proteins/enzymes outside phagolysosomes, especially those which contain methionine in an active center (Table 1), may result in altering their biological functions (Carr et al. 2001; Naskalski et al. 2002).

In the present study we have compared the effect of taurine and TauCl on activity of A1AT, the most abundant inhibitor of serine proteases, with that of HOCl, a physiological inhibitor of A1AT itself (Montecucco et al. 2009; Schönberg et al. 2012). A1AT is a specific endogenous inhibitor of NE showing anti-inflammatory properties. At the same time NE, as well as other proteases, is involved in microbial killing during phagocytosis to play an important role in host defense (Smith 1994). Moreover, NE is required for neutrophil extracellular trap formation confirming its contribution to host defense (Palmer et al. 2012). Therefore, to maintain antibacterial activity of proteases (NE, cathepsin G, proteinase 3), A1AT, their physiological inhibitor, has to be inhibited by HOCl.

On the other hand, inhibition of extracellular A1AT may result in tissue destruction and exacerbation of inflammation due to an imbalance between pro- and anti-

Table 1 HOCl-induced oxidation of proteins. A dose-dependent effect

Target protein	HOCl-protein ratio[a] (fold)
Protein fragmentation	
Bovine serum albumin	>70
Ovalbumin	>50
IgM	>800
Protein (enzyme) inactivation	
Inhibitor of serine proteases	<3
Serine proteases (cathepsin G)	20–50
Catalase	>180
Superoxide dismutase	>750

[a]The extent of protein modification with HOCl is dependent on the molar ratio of HOCl to reactive amino acid side chains

inflammatory mediators (Bergin et al. 2012). All these data indicate that A1AT plays a dual role at a site of inflammation. A1AT unique anti-inflammatory properties justified its therapeutic potential in a number of diseases. To maintain its activity in chronic inflammatory regions A1AT application should be supported by simultaneous usage of HOCl scavengers, such as taurine, methionine, chlorhexidine (Schönberg et al. 2012).

Our present results are in agreement with the previous studies (Bergin et al. 2012). In our experimental system HOCl inhibited the activity of A1AT in a dose dependent manner (Fig. 2b) without any effect on the activity of trypsin (the selected target protease for A1AT) (Fig. 3). Importantly, TauCl, the product of reaction between taurine and HOCl, did not affect A1AT (Fig 2c). It suggests that *in vivo* application of excess of taurine to neutralize detrimental effect of HOCl will retain A1AT activity even after conversion of HOCl to TauCl. Moreover, A1AT together with TauCl will show anti-inflammatory activity.

3.2 Adaptive Immunity: Taurine Blocks the Chlorination of Self- and Non-self-Antigens and Neutralizes "Adjuvant-Like" Effect of HOCl

In our previous studies we have investigated the effect of OVA chlorination on its immunogenicity in both *in vitro* and *in vivo* models of OVA-specific immune responses (Marcinkiewicz et al. 1991, 1992; Olszowski et al. 1996). We have shown that HOCl is a powerful enhancer of antigen processing and presentation. *In vitro*, chlorinated OVA lowered the threshold of ovalbumin required to stimulate OVA-specific T cell hybridoma (Marcinkiewicz et al. 1998). *In vivo*, immunization of mice with chlorinated OVA resulted in an enhanced OVA-specific humoral response (Marcinkiewicz et al. 1992). We have also explained some mechanisms responsible for increased immunogenicity of chlorinated antigens. Primarily, chlorination facilitates antigen processing/presentation enhancing the antigen uptake by APCs and increasing the susceptibility of protein to proteolysis (Marcinkiewicz 1997;

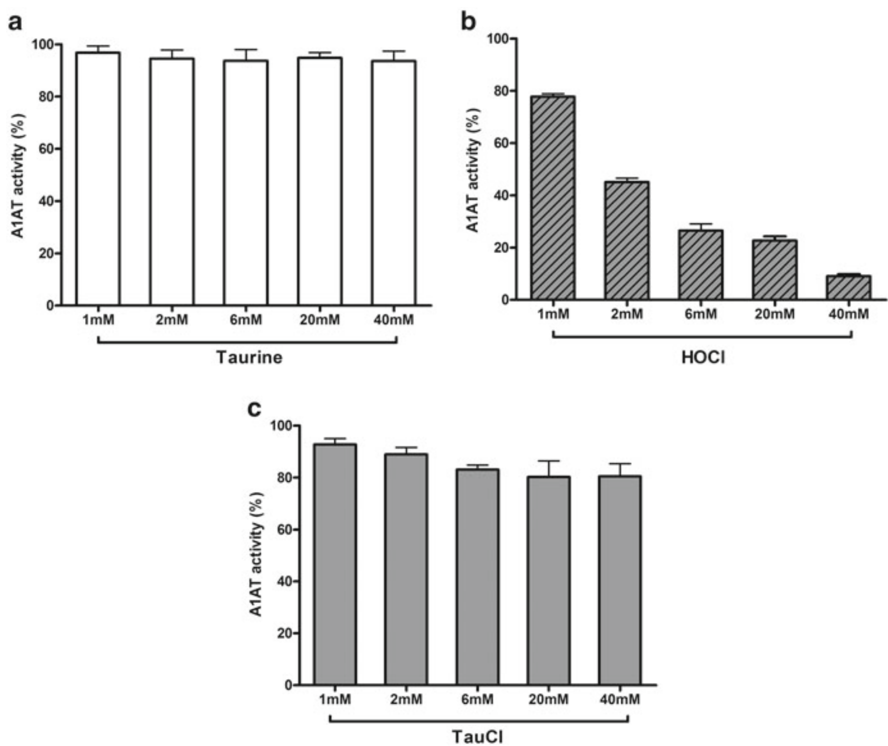

Fig. 2 The effect of taurine (**a**), HOCl (**b**) and TauCl (**c**) on A1AT activity. A1AT activity was measured in an enzymatic assay, as described in Sect. 2

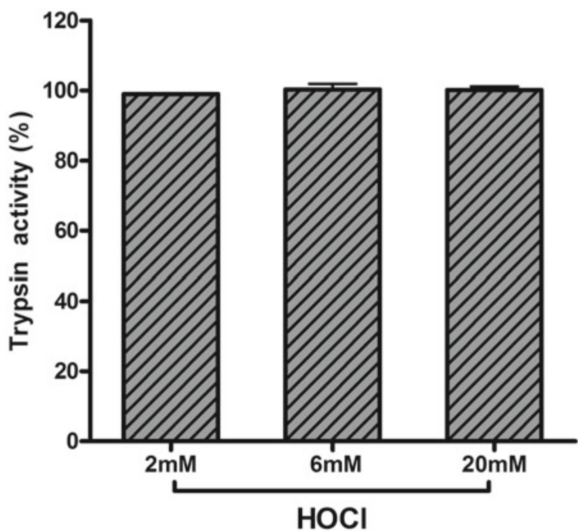

Fig. 3 The effect of HOCl on trypsin activity. Trypsin activity was measured in an enzymatic assay, as described in Sect. 2

Olszowska et al. 1989). Then, we have found that this effect was epitope specific. The positive effect of chlorination (enhancement of T cell and B cell responses) is restricted to epitopes which do not contain the HOCl target molecules such as methionine and cysteine (Prokopowicz et al. 2010).

All these data suggest that HOCl may be used as novel adjuvant to enhance immunity of vaccines. However, still some questions remain open, such as a physiological role of HOCl in modification of antigen immunogenicity. For example, it is not clear whether HOCl, in the presence of taurine, is able to alter immunogenicity of exogenous (non-self) proteins endocytosed by neutrophils *in vivo*. Secondly, is it possible that chlorination of autoantigens occurs as a consequence of inflammation and that increased immunogenecity of chlorinated autoantigens may lead to breaking of tolerance and induction of autoimmune reactions? This hypothesis, formulated by Westman and Harris (2004), was supported by the data showing that rats immunized with chlorinated rat serum albumin (RSA-Cl) developed IgG specific for RSA-Cl which cross-reacted with native RSA.

To investigate these problems we studied the humoral response in mice to native and chlorinated non-self (OVA) and self-antigens (MSA). In addition, the influence of taurine on protein chlorination was determined. We have shown that taurine, when added in excess, significantly blocks HOCl-mediated enhancement of immunogenicity of both tested proteins (Fig. 4 OVA, Fig. 5 MSA). It suggests that high

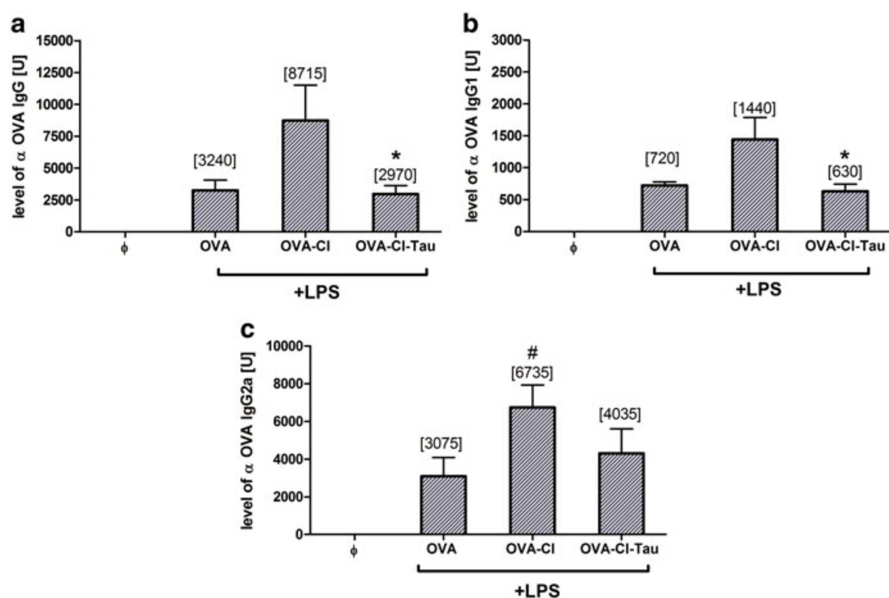

Fig. 4 The effect of OVA chlorination in the presence of taurine on anti-native OVA IgG (**a**), IgG1 (**b**), IgG2A (**c**) production. Mice were immunized (primary immunization) i.p. with OVA+LPS, OVA-Cl or OVA-Cl-Tau. All mice received LPS as an adjuvant. On day 14 mice were rechallenged (boost) with OVA and LPS. Five days later level of IgG, IgG1, IgG2a anti-native OVA was estimated. *$p<0.05$ OVA-Cl vs. OVA-Cl-Tau, #$p<0.05$ OVA vs. OVA-Cl (n=6)

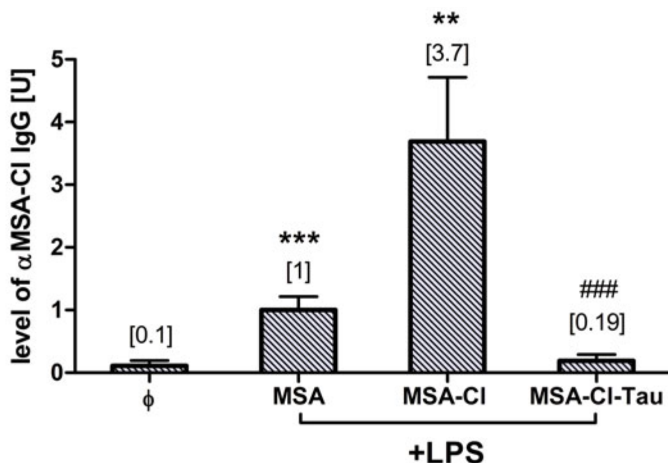

Fig. 5 The effect of MSA chlorination in the presence of taurine on anti-chlorinated MSA IgG production. Mice were immunized (primary immunization) i.p. with MSA+LPS, MSA-Cl or MSA-Cl-Tau. All mice received LPS as an adjuvant. On day 14 mice were rechallenged (boost) with MSA and LPS. Five days later level of IgG anti-chlorinated MSA was estimated. **$p < 0.01$ MSA vs. MSA-Cl, ***$p < 0.001$ ϕ vs. MSA-Cl , ###$p < 0.001$ MSA-Cl vs. MSA-Cl-Tau ($n = 6$)

cytoplasmic concentration of neutrophil taurine prevents breaking tolerance which could occur at a site of inflammation, as an effect of protein chlorination. Therefore, in our opinion, the risk of *in vivo* induction of autoimmunity by HOCl-modified self-antigens is possible but limited. *In vivo*, in contrast to *in vitro* systems, the effective oxidative modification of self-proteins by the MPO-halide system is markedly limited by: trapping free HOCl by taurine, non-effective molar ratio of HOCl to self-proteins and absence of MPO (HOCl) in cytosol of antigen presenting cells (APC).

4 Conclusions

In summary, the reaction of taurine with HOCl results in the formation of TauCl, a less toxic, mild oxidant with anti-inflammatory, pro-apoptotic, and anti-microbial properties. Therefore, the primary role of taurine in innate and adaptive immunity is protecting self-components of the immune system from HOCl-mediated oxidative damage. At the site of inflammation, the conversion of HOCl to TauCl switches neutrophils from pro-inflammatory to anti-inflammatory activity. In adaptive immunity, high concentration of taurine in leukocytes will eliminate “adjuvant-like effect” of HOCl against self-proteins to limit the risk of autoimmune reactions.

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Taurine Haloamines and Biofilm. Part I: Antimicrobial Activity of Taurine Bromamine and Chlorhexidine Against Biofilm Forming *Pseudomonas aeruginosa*

Magdalena Strus, Maria Walczewska, Agnieszka Machul, Diana Mikołajczyk, and Janusz Marcinkiewicz

Abbreviations

CFU	Colony forming units
CHX	Chlorhexidine
CIP	Ciprofloxacin
HOBr	Hypobromous acid
LCL	Luminol dependent chemiluminescence
OZ	Opsonized zymosan
P. aeruginosa	*Pseudomonas aeruginosa*
PMN	Peritoneal mouse neutrophils
ROS	Reactive oxygen species
Tau	Taurine
TauBr	Taurine bromamine

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1 Introduction

Resistance to antimicrobial agents is the most important feature of biofilm-associated chronic infections, such as chronic rhinosinusitis, periodontal diseases and chronic non-healing wounds (Høiby et al. 2010). Especially, chronic wounds infected by *Pseudomonas aeruginosa* remain a significant medical burden in human and veterinary medicine of today (Bjarnsholt et al. 2008; Westgate et al. 2011; Wilkins and Unverdorben 2013). It is well documented that *P. aeruginosa* biofilm is highly resistant to a variety of antibiotics. Among them is ciprofloxacin, the most commonly used antibiotic for treatment of *P. aeruginosa* infections (Morita et al. 2014). Importantly, the formation of static biofilms increases when *P. aeruginosa* cells are exposed to sub-inhibitory concentrations of some antibiotics, including ciprofloxacin (Linares et al. 2006). In addition to antibiotic-resistance, bacteria hidden in a biofilm matrix are able to avoid phagocytosis and may even kill neutrophils (Leid et al. 2005). Thus, the anti-biofilm host defense becomes compromised in spite of high antimicrobial potential of neutrophil MPO-halide system (Jesaitis et al. 2003).

The MPO-halide system of neutrophils generates hypochlorous (HOCl), and hypobromous acid (HOBr), the strong microbicidal oxidants, in phagolysosomes (Thomas et al. 1995; Gaut et al. 2001). In turn, the excess of HOCl and HOBr reacts with taurine, the most abundant free amino acid in neutrophil cytosol, to form less toxic taurine chloramine (TauCl) and taurine bromamine (TauBr), respectively (Henderson et al. 2001). TauCl and TauBr themselves show antimicrobial properties against a wide range of bacteria, tested *in vitro* mainly against their planktonic form (Nagl et al. 2000; Marcinkiewicz et al. 2005, 2006). Much less is known about their anti-biofilm properties. However, a number of clinical studies have shown that both haloamines are effective in the local treatment of skin and mucosa infections, including biofilm-related infections (Nagl et al. 2000; Marcinkiewicz et al. 2008; Marcinkiewicz 2009). Moreover, we have also shown recently that taurine haloamines can inhibit the formation of *P. aeruginosa* biofilm *in vitro* at its early stages of the development. These preliminary data suggest that TauBr is a good candidate for a local therapy against biofilm-related infections (Marcinkiewicz et al. 2013a). However, a combined therapy, an application of TauBr together with other anti-biofilm agents, seems to be more promising. One of them is chlorhexidine (CHX), the anti-biofilm agent that has been tested in combination with a number of other antiseptics (e.g. hydrogen peroxide, sodium hypochlorite) but never with taurine haloamines (Bonez et al. 2013; Montecucco et al. 2009).

The aim of this study was to extend the investigations concerning the capacity of TauBr to inhibit formation of *P. aeruginosa* biofilm. In our experimental set-up a planktonic form and mature biofilm was treated *in vitro* with TauBr alone or TauBr and ciprofloxacin. Efficacy of TauBr was compared with that of CHX, the most effective anti-biofilm agent that has been developed to date. Moreover, the impact of TauBr and CHX on the production of reactive oxygen species (ROS) by activated neutrophils was investigated to compare anti-oxidant potential of the tested agents.

2 Methods

2.1 Chemicals

Taurine, Chlorhexidine digluconate (CHX), luminol sodium salt, zymosan A, Ciprofloxacin (Sigma-Aldrich, Germany).

2.2 Preparation of Taurine Bromamine

Taurine bromamine (TauBr) was prepared by a reaction of taurine with HOBr as published previously (Marcinkiewicz et al. 2006). First, HOBr was synthesized in reaction between equimolar amounts of HOCl and NaBr (POCH, Poland) in PBS solution. In such conditions virtually all the OCl^- present reacts with Br^- to form OBr^- and Cl^-.The presence and concentration of OBr^- was confirmed by UV spectra ($\lambda = 200–400$ nm). In the second step, 20 mM HOBr was added dropwise to equal volume of 400 mM taurine. UV absorption spectrum was checked to exclude the formation of taurine dibromamine or chloramines and to estimate the concentration of TauBr (molar extinction coefficient $e = 430\ M^{-1}\ cm^{-1}$ at A_{288}). Stock solution of TauBr was kept at 4 °C for a maximum period of 3 days before use.

2.3 Microbial Strains and Culture Conditions

All experiments were performed on *P. aeruginosa* strain coded as PAR 5 which was isolated from suppurating wound of patients with chronic diabetic foot infection. The strain was propagated in 10 ml of Tryptic Soy Broth (TSB, Difco) at 37 °C for 24 h at aerobic conditions. Then the culture was centrifuged (2,000 rpm; 10 min) and washed with 10 ml of NaCl 0.9 %. Initial suspension of the strain (1×10^8 CFU/ml) was prepared by making an appropriate dilution of the bacteria in saline. Cell numbers were checked using MacFarland's scale and by counting bacterial colonies plated from decimal dilutions on McConkey Agar (Oxoid).

2.3.1 Determination of Ciprofloxacin Inhibitory Activity Against Planktonic *Pseudomonas* Culture

Determination of ciprofloxacin inhibitory activity against planktonic culture for PAR 5 strain was checked in a 96-well plastic plate (Greiner Bio-One, Germany). To each well 180 μl fresh TSB broth and 20 μl of the 24 h-old culture of PAR 5 were added. Then, the plate was centrifuged (2,000 rpm; 10 min) to adhere bacteria to the bottom of the plate and 20 μl of culture medium was removed from each well and the same volume of chemically pure ciprofloxacin (CIP, Sigma-Aldrich) was added.

The following concentrations of ciprofloxacin were tested: 1; 8; 32 μg/ml. The plates were then gently stirred to distribute the antibiotic in the wells and culture was continued at 37 °C for 24 h at aerobic conditions. Three independent cultures were made for each concentration of CIP. Density of the bacterial populations was tested separately after 4, 8, 24 h of incubation. Numbers of viable bacteria were estimated by making serial decimal dilutions in saline of bacteria removed from the bottoms of the wells by multiple pippeting and plating them on McConkey Agar (Oxoid). The final result was presented in CFU/ml using a standard viable count technique.

2.3.2 Determination of Ciprofloxacin (CIP) Inhibitory Activity Against 24 h-Old *Pseudomonas* Cells Embedded in the Biofilm Matrix (Mature Biofilm)

For this purpose, 180 μl of TSB broth and 20 μl of the 24 h-old culture of PAR 5 were added to each well of a 96-well plastic plate and, as above, the plate was centrifuged (2,000 rpm; 10 min) to adhere the bacteria to the bottom of the plate and incubated for 24 h allowing mature biofilm formation. Afterwards, 20 μl of the culture was removed from each well and the same volume of an appropriate grading concentration of chemically pure ciprofloxacin (Sigma-Aldrich) was added to the well. The following concentrations of ciprofloxacin were tested: 1; 4; 8 μg/ml. Bacterial culture was dispensed into three wells, because populations of bacteria were tested separately for each concentration of CIP after 4, 8, 24 h of incubation. The plates were then gently stirred to distribute the antibiotic in the wells, and the culture was continued at the same conditions. Numbers of viable bacteria were verified by using the same methods as described above.

2.3.3 Effects of TauBr, CHX and Ciprofloxacin on the Number of Viable Bacteria Growth in Two Different Phases (Planktonic and Mature Biofilm)

Inhibitory activity of TauBr, CHX and CIP against planktonic *PAR 5* growth was checked by filling wells in a 96-well plate with 20 μl of *PAR 5* suspension containing 1×10^8 bacteria/ml of the test strain and 180 μl of fresh TSB, then in the same way as described above, the plate was centrifuged. TauBr, CHX and CIP solutions were added to obtain their final concentration of 300 μM for TauBr, 0.2 % for CHX and 8 μg/ml for CIP. Afterwards the plates were gently mixed and incubated for 24 h at 37 °C. Counting of the bacteria was done in the following time intervals (0, 4, 8, 24 h). The results were calculated in CFU/ml.

Effects of TauBr, CHX and CIP on already formed mature biofilm of *P. aeruginosa* were measured in the same way but the test substances at final concentration were added after biofilm formation i.e. in 24 h after adherence of the bacteria to the wells. The plates were then incubated as above and biofilm and bacterial numbers were measured in the following time intervals (24, 28, 32, 48 h). The results were calculated in CFU/ml.

2.4 Isolation of Neutrophils

Peritoneal mouse neutrophils (PMN) isolated from CBA mice were induced by intraperitoneal injection of 1.0 ml of thioglycolate. Cells were collected 18 h later by washing out the peritoneal cavity with 5 ml of PBS (phosphate buffer solution) containing 5 U heparin/ml Cells were centrifuged and red blood cells were lysed by osmotic shock using distilled water; osmolarity was restored by addition of 2× concentrated PBS.

2.5 Measurement of ROS Production by Activated Neutrophils Using Luminol-Dependent Chemiluminescence (LCL)

Mouse peritoneal exudate neutrophils were incubated with TauBr (100–300 μM) or CHX (0.001–0.00025 %) in the presence of luminol (0.8 mg/ml) for 30 min. The cells were then stimulated with opsonized zymosan (OZ) (0.4 mg/ml) and immediately the photon emission was measured in temperature-stabilized luminometre Lucy 1 over 75 min with 3-min intervals. Results are expressed as relative light unit (RLU).

3 Results

Inhibitory activity of ciprofloxacin against culture of *P. aeruginosa* depends mainly on the two factors: the concentration of antibiotic and the phase of bacterial growth (Tables 1 and 2).

Table 1 Inhibitory activity of ciprofloxacin against planktonic form of *P. aeruginosa*

	0 h	4 h	8 h	24 h
Culture control PAR 5	1×10^7	5×10^7	2×10^8	1×10^8
Ciprofloxacin [1 μg/ml]	2×10^6	3×10^4	1×10^4	7×10^3
Ciprofloxacin [8 μg/ml]	3×10^5	0	0	0
Ciprofloxacin [32 μg/ml]	1×10^4	0	0	0

Table 2 Inhibitory activity of ciprofloxacin against 24 h-old *P. aeruginosa* cells embedded in the biofilm matrix (mature biofilm)

	24 h	24+4 h	24+8 h	24+24 h
Culture control PAR 5	1.2×10^8	1×10^8	2×10^8	1.5×10^8
Ciprofloxacin [1 μg/ml]	3×10^8	5×10^7	4×10^6	2×10^5
Ciprofloxacin [8 μg/ml]	4×10^7	4×10^4	3×10^4	1×10^2
Ciprofloxacin [32 μg/ml]	1×10^7	2×10^2	1×10^1	0

Table 3 Effect of chlorhexidine on the number of *P. aeruginosa* bacteria [CFU/ml] in planktonic form

	0 h	4 h	8 h	24 h
Culture control	6×10^6	8×10^7	4×10^8	3.5×10^8
Chlorhexidine (0.2 %)	2.5×10^5	0	0	0
Chlorhexidine (0.2 %) and ciprofloxacin [8 μg/ml]	2×10^5	0	0	0
Ciprofloxacin [8 μ/ml]	3×10^5	0	0	0

Table 4 Effect of TauBr on the number of *P. aeruginosa* bacteria [CFU/ml] in planktonic form

	0 h	4 h	8 h	24 h
Culture control	2×10^7	1.5×10^7	2×10^8	4×10^8
TauBr (300 μM)	1×10^5	0	0	0
TauBr (300 μM) and ciprofloxacin [8 μg/ml]	2×10^5	0	0	0
Ciprofloxacin [8 μg/ml]	4×10^5	0	0	0

In case of planktonic form the total reduction of PAR5 population was observed after 4 h by 8 and 32 μg/ml CIP concentration. In mature biofilm on the other hand, decrease in the number of viable bacteria to zero was reported only for CIP 32 μg/ml, after 24 h of addition of the antibiotic to the matrix of the biofilm. For a combined antibacterial effect of TauBr and CHX only 8 μg/ml CIP concentration as a control was chosen.

3.1 *Effect of TauBr, CHX and CIP on the Number of Viable* **P. aeruginosa** *Bacteria in Planktonic Form*

In our experimental set up TauBr, CHX and CIP killed 100 % bacterial cells after 4 h incubation. The bactericidal effect was observed for substances alone or in combination (Tables 3 and 4). This part of experiments confirms microbicidal potential against planktonic form of *P. aeruginosa* bacteria of all testes agents.

3.2 *Effect of TauBr, CHX and Ciprofloxacin on the Number of Viable* **P. aeruginosa** *Bacteria in the Mature Biofilm*

The effect of the tested substances on the mature biofilm confirmed that bacteria hidden in matrix are more resistant to antibacterial agents then their planktonic form. Only CHX killed all of them after 4 h incubation. On the contrary, TauBr had no effect on the mature biofilm, as the number of live bacterial cells was not reduced. CIP antibacterial effect was also significantly weaker compared with CHX. Incubation times of 4 h were needed for a 3 logarithms reduction of *P. aeruginosa* (Tables 5 and 6—mature biofilm). Additive or synergistic effects of these agents were not observed.

Table 5 Effect of chlorhexidine on the number of *P. aeruginosa* bacteria [CFU/ml] in the mature biofilm

	24 h	28 h	32 h	48 h
Culture control PAR 5	3×10^7	4×10^8	3×10^8	3×10^8
Chlorhexidine (0.2 %)	2×10^6	0	0	0
Chlorhexidine (0.2 %) and ciprofloxacin [8 μg/ml]	1×10^6	0	0	0
Ciprofloxacin [8 μg/ml]	4×10^7	4×10^4	3×10^4	1×10^2

Table 6 Effect of TauBr on the number of *P. aeruginosa* bacteria [CFU/ml] in the mature biofilm

	24 h	28 h	32 h	48 h
Culture control PAR5	2.5×10^8	5×10^8	3×10^8	2×10^8
TauBr (300 μM)	1.5×10^8	1×10^8	1×10^8	2×10^8
TauBr (300 μM) and ciprofloxacin [8 μg/ml]	2×10^6	3×10^4	2×10^4	8×10^2
Ciprofloxacin [8 μg/ml]	4×10^7	4×10^4	3×10^4	1×10^2

3.3 The Effect of TauBr and CHX on the ROS Production by Neutrophils Stimulated with Opsonized Zymosan

To determine anti-oxidant potential of TauBr and CHX, the agents were added to zymosan activated neutrophils and the release of ROS was measured by luminol-dependent chemiluminescence (LCL). As shown in Fig. 2, TauBr, used at non-cytotoxic concentrations (100–300 μM), decreased the LCL in a dose dependent manner. Surprisingly, the effect of CHX was opposite. CHX markedly enhanced the zymosan-induced LCL even at the lowest concentration used. However, CHX did not induce LCL in resting neutrophils (data not shown).

4 Discussion

P. aeruginosa is an opportunistic pathogen, a common causative agent of a variety of infections associated with biofilm formation, including respiratory tract and chronic wound infections (Drenkard 2003). The effectiveness of treatment of such infections is very often hampered by antibiotic resistance. *P. aeruginosa* itself readily acquires resistance to individual agents via chromosomal mutations and lateral gene transfer (Morita et al. 2014). Moreover, when sessile bacterial cells start to produce biofilm matrix components they become protected from antibiotic and immune attack (Ryder et al. 2007). In addition, it has been shown that sub-inhibitory concentration of many antibiotics, including ciprofloxacin, increases the formation of thicker and more robust *P. aeruginosa* biofilm (Linares et al. 2006). Therefore, to improve effectiveness of antibiotic therapy in *P. aeruginosa* chronic infections, a variety of novel anti-biofilm agents have been tested (e. g DNase, taurolidine, sodium hypochlorite, hydrogen peroxide, chlorhexidine) (DeQueiroz and Day 2007; Walczewska et al. 2013). These anti-biofilm drugs which are able to penetrate

and destroy the components of biofilm matrix and kill hidden bacteria, should be applied either before or along with antibiotics. Such therapeutic strategies could be an interesting option for biofilm elimination and should diminish the risk of inappropriate chemotherapy which readily selects multidrug-resistant *P. aeruginosa* (Morita et al. 2014).

The results from our previous studies suggest that taurine haloamines, especially TauBr, are promising candidates for such therapy (Marcinkiewicz et al. 2013b). TauBr was effective in the local treatment of inflammatory *acne vulgaris*, a biofilm-related inflammatory skin disease (Marcinkiewicz et al. 2006, 2008). Moreover, we have recently reported that TauBr, but not TauCl, when used at low concentrations (300 and 1,000 μM) is able to inhibit the formation of *P. aeruginosa* biofilm *in vitro* (Marcinkiewicz et al. 2013a). Our observations concerning the activity of TauCl are contradictory to the recent report of Coraça-Huber et al. (2014). They found a time- and concentration-dependent anti-biofilm activity of TauCl. However, they used TauCl at very high concentrations between 5.5 and 55 mM, which markedly exceeded the physiological concentration of TauCl and being cytotoxic when tested *in vitro*. Surprisingly, it is well documented that TauCl can be applied clinically to different body regions and cavities at such high concentrations without detrimental side effects (Gottardi and Nagl 2010).

In the present study, we have focused on TauBr to examine anti-biofilm properties of novel candidates for the local treatment of non-healing wounds. TauBr antibacterial and anti-inflammatory (anti-oxidant) properties were compared with that of chlorhexidine (CHX), a broad spectrum antiseptic (Bonez et al. 2013). The choice of CHX is justified by a fact that it is the best studied antibiofilm agent to date. More importantly, CHX is one of the most frequently used antiseptics, effective against developing and mature biofilms, and in dentistry is treated as “the golden standard” for oral cavity antiseptic procedures (Hübner et al. 2010).

To compare therapeutic antibacterial/antibiofilm potential of TauBr, CHX and ciprofloxacin we used our *in vitro* experimental model of *P. aeruginosa* biofilm Fig. 1 (Marcinkiewicz et al. 2013a). As expected, in the early biofilm, all tested agents (ciprofloxacin, TauBr and CHX), either alone or in combination, killed all bacterial cells (Tables 1, 2, and 3—early biofilm). Therefore, the present study confirms microbicidal potential of all tested agents when used against planktonic form of bacteria. However, when the agents were tested against sessile bacteria hidden in a biofilm matrix (Tables 4 and 5—mature biofilm), only CHX retained its bactericidal/anti-biofilm properties. The bactericidal effect of CHX was immediate. On the contrary, TauBr used at the bactericidal concentrations against planktonic form of *P. aeruginosa*, had no effect on the mature biofilm, as the number of live bacterial cells was not reduced. Also ciprofloxacin antibacterial effect was significantly weaker and delayed as compared its effect against planktonic form of bacteria. These data suggest that TauBr alone is not able to kill bacteria hidden in a biofilm, while ciprofloxacin needs to be used at much higher concentrations than its MIC determined against planktonic form of *P. aeruginosa.* Therefore, we ask the question whether TauBr is able to enhance the efficacy of ciprofloxacin. Additive or synergistic effect of these agents would be clinically important as it

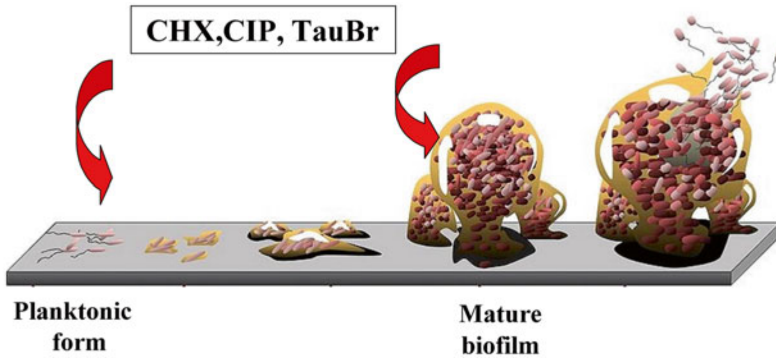

Fig. 1 Development of *P. aeruginosa* biofilm and visualization of the application of TauBr, CHX and CIP at two different phases of bacterial growth

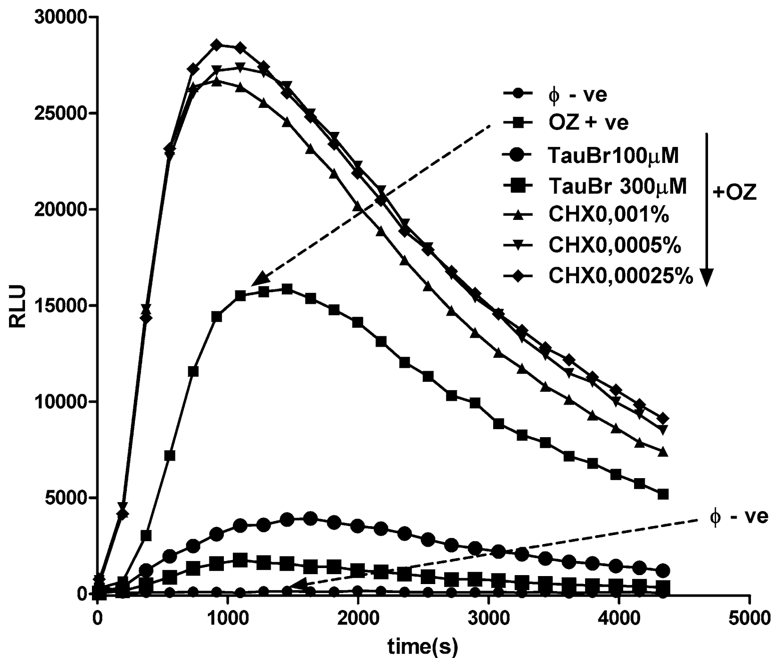

Fig. 2 Effect of CHX and TauBr on the ROS production by neutrophils stimulated with opsonized zymosan. Neutrophils (5×10^5/well) were incubated with CHX or TauBr. LCL was then performed and measured as described in methods. ϕ-nonstimulated cells, negative control; OZ-stimulated cells, positive control (LCL = 100 %). The figure shows one representative experiment

could reduce the risk of non-effective monotherapy (ciprofloxacin) and the selection of resistant *P. aeruginosa* strains. Unfortunately, there was no additive effect between TauBr and ciprofloxacin. These *in vitro* results are contradictory to the

therapeutic effect of TauBr in the local treatment of inflammatory acne lesions (Marcinkiewicz 2009). In our pilot clinical study we have compared the efficacy of TauBr with Clindamycin, one of the most common topical agents in the treatment of acne. Both agents produced comparable beneficial results (Marcinkiewicz et al. 2008). This therapeutic effect of TauBr may be explained by its strong anti-inflammatory properties in addition to its ability to selectively kill *Propionibacterium acnes*, a pathogenic factor of *acne vulgaris* (Marcinkiewicz et al. 2006). Moreover, TauBr reduces the generation of reactive oxygen species (especially H_2O_2) from activated neutrophils, which seems to be crucial for decreasing the number and severity of inflammatory lesions in patients with mild acne. Importantly, antibiofilm properties of TauBr against two different bacteria species, *P. acnes* and *P. aeruginosa*, should not be compared due to distinct composition of their biofilm matrix (Ghafoor et al. 2011; Whitchurch et al. 2002).

Further, we have compared anti-inflammatory (anti-oxidant) properties of TauBr and CHX. We have examined their ability to reduce ROS generation by activated neutrophils. The results clearly indicate that TauBr can neutralize or reduce generation of ROS. Surprisingly, CHX enhanced the level of ROS. It suggests that CHX may exhibit both antioxidant and pro-oxidant properties under different conditions. Indeed, CHX was shown to be a mild scavenger of hydroxyl radicals generated in a Fenton reaction. On the other hand, CHX induced ROS production in the presence of calcium hydroxide (Yeung et al. 2007). However, further studies are necessary to explain the mechanism of this dual effect of CHX. In summary, all these data suggest that in spite of superior antibiofilm activity of CHX compared to TauBr, both compounds may be used either alternatively or in a combination in the local treatment of chronic inflammatory infections to inhibit biofilm formation and to facilitate its elimination.

5 Conclusions

The present data confirm strong bactericidal properties of CHX and TauBr against planktonic form of *P. aeruginosa* and show that TauBr and CHX inhibit the formation of early biofilm *in vitro*. However, only CHX destroys the preformed biofilm and effectively kills sessile bacterial cells. On the other hand, TauBr, but not CHX, shows antioxidant properties. Although we have not found additive antibacterial effect of ciprofloxacin, CHX and TauBr, we suggest that CHX (antimicrobial and anti-biofilm agent) and TauBr (antimicrobial and antioxidant agent) may be combined in therapy of chronic infections associated with biofilm formation and generation of oxidative stress for their complementary therapeutic actions. Such novel therapeutic strategy could be used not only in treatment of chronic skin infections but also in periodontal diseases associated with excessive dental plaque formation.

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Taurine Haloamines and Biofilm: II. Efficacy of Taurine Bromamine and Chlorhexidine Against Selected Microorganisms of Oral Biofilm

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Abbreviations

C. albicans	*Candida albicans*
CFU	Colony forming unit
CHX	Chlorhexidine
HOCl	Hypochlorous acid
MIC	Minimal inhibitory concentration
NaOCl	Sodium hypochlorite
P. gingivalis	*Porphyromonas gingivalis*
S. mutans	*Streptococcus mutans*
TauBr	Taurine bromamine
TauCl	Taurine chloramine

1 Introduction

Uncontrolled bacteria of dental plaque are responsible for the formation of oral biofilm located on teeth and subgingival surfaces (Kolenbrander et al. 2002; Demling et al. 2009). Orthodontic appliances, such as metal, ceramic brackets and composite materials, facilitate bacterial adhesion and accelerate oral biofilm

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formation (Lindel et al. 2011; Papaioannou et al. 2007, 2012). It may induce local inflammation (gingivitis) with further development of periodontal diseases (Sbordone and Bortolaia 2003). Very often oral bacterial infections are associated with candidiasis (Williams and Lewis 2011).

Chlorhexidine digluconate (CHX) is the most commonly used disinfectant and topical antiseptic in dentistry. It is the best anti-plaque agent that has been developed to date (Guggenheim and Meier 2011; Bonez et al. 2013; Gomes et al. 2013). CHX acts bacteriostatically at low concentrations, while at higher concentrations it can kill bacterial cells. However, CHX has some well-known adverse effects, such as yellow staining of teeth, allergy and cytotoxicity to odontoblasts (Lessa et al. 2010). Moreover, interaction between CHX and sodium hypochlorite, an irrigant used in root canal treatment, may result in formation of para-chloroaniline, a toxic and carcinogenic agent (Basrani et al. 2007). Thus, due to CHX adverse effects, there is ongoing search for alternative antiseptics. One of these novel antiseptics in dentistry is taurolidine (Eick et al. 2012). Taurolidine, a derivative of free amino acid taurine, has a significant effect on supragingival plaque biofilm, although not as pronounced as that of CHX (Arweiler et al. 2012). These data clearly support our idea to examine anti-plaque properties of taurine haloamines, taurine chloramine (TauCl) and taurine bromamine (TauBr), the agents with known antimicrobial properties against various pathogens (Mainnemare et al. 2004; Marcinkiewicz et al. 2006, 2013a; Pasich et al. 2013a). Moreover, clinical studies have shown that both haloamines are effective in the local treatment of skin and mucous infections, including biofilm-related infections (Coraça-Huber et al. 2014; Marcinkiewicz et al. 2008, 2013b; Nagl et al. 2000). However, up to date no studies were performed on their ability to kill bacteria hidden in dental plaque biofilm or disrupt oral biofilm structure. The aim of this study was to examine ability of TauBr to kill the selected oral microorganisms, hidden in a dental biofilm. First, we have developed a laboratory biofilm plaque model system formed on various orthodontic materials. Then, we have compared the microbicidal activity of CHX and TauBr against biofilm forming *Streptococcus mutans* and *Candida albicans* grown on the selected orthodontic material (composite discs).

2 Methods

2.1 Chemicals

Taurine, chlorhexidine digluconate (CHX), hypochlorous acid (HOCl), mucin were obtained from Sigma-Aldrich (Germany). Tryptic soy agar (TSA), Tryptic soy broth (TSB), Sabouraud Agar from Heipha Diagnostica (Germany).

2.2 Preparation of TauBr

TauBr was prepared as described in "Taurine Haloamines and Biofilm. Part I: Antimicrobial Activity of Taurine Bromamine and Chlorhexidine Against Biofilm Forming *Pseudomonas aeruginosa*". UV absorption spectrum was checked to exclude the formation of taurine dibromamine and to estimate the concentration of TauBr (molar extinction coefficient $\varepsilon = 430\ M^{-1}\ cm^{-1}$ at A_{288}). Stock solution of TauBr was kept at 4 °C for a maximum period of 3 days before use.

2.3 Microbial Strains and Culture Conditions

Streptococcus mutans ATCC 25175 was grown in an aerobic atmosphere in TSA and 5 % sheep blood for 72 h before tests. *Candida albicans* ATCC 90029 was grown in aerobic conditions in Sabouraud Agar for 48 h before tests.

2.4 Measurement of Microbicidal Activity of TauBr and CHX Against Planktonic Form of S. mutans and C. albicans

MIC was determined by the pour plate method. The agents (TauBr, CHX), used at different concentrations, were incubated in low (10^5/ml) or high (10^8/ml) density microbial suspensions for 1 h, if not otherwise stated. Then aliquots of these dilutions were spread on growing agar plates and colony forming units (CFU) were counted after 48 h.

2.5 Experimental Model of Biofilm Formation on the Different Types of Orthodontic Materials

Plates (size 5×5mm) made of different orthodontic materials: acryl, cobalt, nickel and composite were used. Briefly, mucin covered plates were incubated with 100 μl of *S. mutans* or *C. albicans* suspensions prepared at the density of 10^5 CFU/ml. After 2 or 24 h plates were washed with 0.9 % NaCl to eliminate unbound bacterial cells, then the plates were blotted onto a contact solid medium (TSA) and cultured for additional 48 h. Then, CFU were counted. In our experimental conditions the number of surviving microbes (*S. mutans*, *C. albicans*) isolated from 24 h biofilm was approximately 400 CFU/25 mm^2 (>300 CFU/plate). Adhesion of microbes and biofilm formation on the plates was visualized using SEM (Scanning Electron Microscopy), as described previously (Głowacki et al. 2014).

2.6 *Measurement of Microbicidal Activity of TauBr and CHX Against* S. mutans *and* C. albicans *Hidden in a Biofilm*

S. mutans or *C. albicans* growing on the composite plates for 2 or 24 h were treated with different concentrations of the tested compounds. After the indicated incubation time (see legends) plates were washed with 0.9 % NaCl and blotted on solid contact medium TSA to grow surviving bacteria. After the next 48 h colonies of *S. mutans* or *C. albicans* were counted and their number (CFU/plate) was compared with the number of colonies isolated from the control plates (non-treated biofilms).

3 Results and Discussion

3.1 *Experimental Models of* S. mutans *and* C. albicans *Biofilms Grown on Different Orthodontic Materials*

Clinical studies have shown that orthodontic therapy using fixed appliances induces adverse effects due to microbial adhesion and increase in oral biofilm formation (Lindel et al. 2011). It raises a question about the best antiseptics used in preventing bacterial attachment and treating oral cavity infections and periodontal diseases. Numerous comparative analyses of bacterial adhesion to wide spectrum of orthodontic materials have shown contradictory results in their ability to form biofilm (Fournier et al. 1998; Demling et al. 2009; van Gastel et al. 2009). In our study, we have examined adhesive properties of the following materials: acryl, cobalt, nickel and composite. To create experimental conditions similar to these in oral cavity, the plates were covered with mucin, a substitute of salivary components important in early pellicle formation on bracket materials (Eliades et al. 1995; Hahnel et al. 2008). Indeed, such pretreatment enhanced the number of bacteria attached to the tested materials in our experimental system, as was described before (Ahn et al. 2002). Importantly, we observed adhesion of *S. mutans* and biofilm formation only on composite plates (Table 1, Fig. 1). In contrast, *C. albicans* cells attached to all tested materials without significant differences (Table 2). Importantly, when the plates were pre-incubated with *C. albicans* cells, we have observed attachment of *S. mutans* to all materials, with preference to composite plates (Table 3). All these data indicate that composite shows the highest adhesive properties for dental plaque microbes, and it suggests that composite appliances create a high risk of periodontal diseases. Therefore, to examine *in vitro* anti-biofilm properties of TauBr against bacteria attached to orthodontic brackets we have chosen composite material (Fig. 2).

Table 1 Adherence of *S. mutans* to different types of orthodontic materials (acryl, cobalt, nickel, and composite plates)

Type of material	*Streptococcus mutans* (CFU/plate)	
	2 h	24 h
Acryl	0	0
Cobalt	0	0
Nickel	0	0
Composite	146 ± 5	>300

The plates were incubated with *S. mutans* suspension (density 10^5 CFU/ml) for 2 and 24 h. The results show the number of live bacteria isolated from plates (CFU/plate). Results depict mean values ± SD calculated from three independent experiments

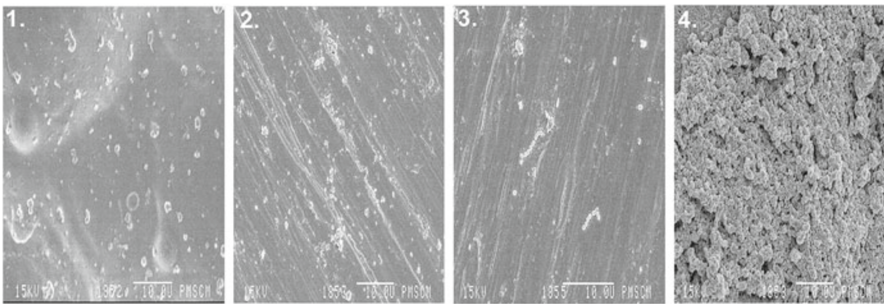

Fig. 1 Biofilm formation on different orthodontic materials. Adherence of *S. mutans* to the selected orthodontic materials (*1*) acryl, (*2*) cobalt, (*3*) nickel, (*4*) composite, after the incubation with a suspension of *S. mutans* (10^5 CFU/ml) for 24 h. The image of SEM (magnification 2,200×)

Table 2 Adherence of *C. albicans* to different types of orthodontic materials/plates

Type of material	*Candida albicans* (CFU/plate)	
	2 h	24 h
Acryl	164 ± 52	>300
Cobalt	209 ± 87	>300
Nickel	108 ± 80	>300
Composite	179 ± 60	>300

The plates were incubated with *C. albicans* suspension (density 10^5 CFU/ml) for 2 and 24 h. The results show the number of live candida cells isolated from the plates (CFU/plate). Results depict mean values ± SD calculated from three independent experiments

Table 3 Adherence of *S. mutans* to different types of orthodontic materials/plates after pre-incubation of the plates with *C. albicans*

Type of material	*Streptococcus mutans* (CFU/plate)
Acryl	52 ± 12
Cobalt	65 ± 10
Nickel	53 ± 29
Composite	>300

First, the plates were incubated with *C. albicans* suspension (density 10^5 CFU/ml) for 24 h and then were incubated with *S. mutans* suspension (density 10^5 CFU/ml) for the next 24 h. The results show the number of live bacteria isolated from the plates (CFU/plate). Results depict mean values ± SD calculated from three independent experiments

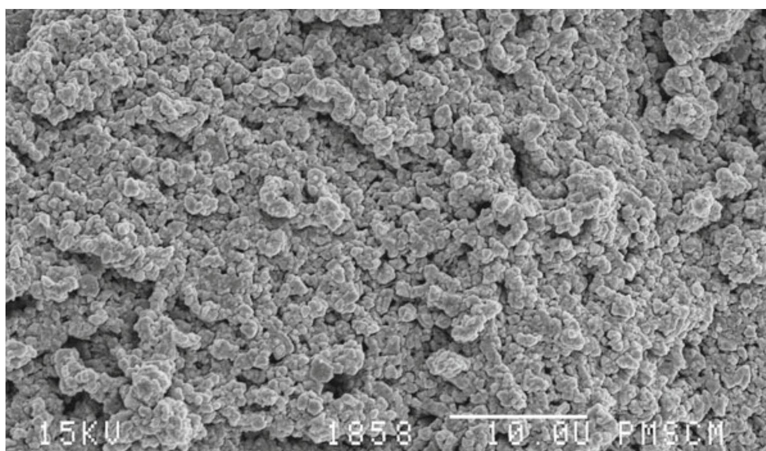

Fig. 2 The image of Scanning Electron Microscopy of 24 h *S. mutans* biofilm on composite plates (magnification 2,200×) (Zagorska—Swiezy K from Department of Otolaryngology CMUJ Krakow)

3.2 Antimicrobial Activity of TauCl, TauBr and CHX Against Planktonic Form of Oral Microbiota

In the previous study we have examined the efficacy of taurine haloamines (TauCl, TauBr) against selected oral microbiome species (*S. mutans*, *P. gingivalis*, *C. albicans*) (Pasich et al. 2013a, b). The effect was compared with that of CHX. All experiments were performed *in vitro* using planktonic form of the tested microbes. *S. mutans* has been selected as the primary bacterial strain isolated from a dental plaque (Hojo et al. 2009). *P. gingivalis* represents anaerobic oral microbiota but is also involved in pathogenesis of periodontal diseases (Stoodley et al. 2002). Finally, *C. albicans* is the most common fungal pathogen associated with oral cavity bacterial infections (Williams and Lewis 2011). In our experimental model CHX showed the strongest antimicrobial activity

Table 4 Serum influence on the microbicidal activity of CHX, TauBr and TauCl against planktonic form of *C. albicans* and *S. mutans*

Tested compound	MIC (%)			
	Candida albicans		*Streptococcus mutans*	
	NaCl (%)	Serum (%)	NaCl (%)	Serum (%)
CHX	0.0015	0.015[a]	0.00015	0.00015
TauBr	0.05	0.11	0.00014	0.055[a]
TauCl	0.18	0.18	0.022	0.045

CHX (0.00002–1.0 %), TauBr and TauCl (0.00001–0.2 %) were incubated for 1 h with the test microorganisms in 0.9 % NaCl alone or in 0.9 % NaCl with 25 % serum. The results represent the average MIC of three independent experiments

[a]Loss of microbicidal activity >10×. The table was prepared basing on data published previously (Pasich et al. 2013a)

against all tested pathogens. On the contrary, TauCl was the weakest antiseptic used, without any effect on the growth of *C. albicans*. These results are contradictory to data published by Nagl et al. (2000). However, they used TauCl at much higher concentrations and for a longer incubation time. Moreover, we have tested effectiveness of our antiseptics against higher density of planktonic pathogens (10^8 vs. 10^5 cells/ml). In contrast to TauCl, TauBr markedly inhibited the growth of *S. mutans* and *P. gingivalis* with a slight effect on the low density of *C. albicans*. Importantly, estimated MIC of TauBr was below its cytotoxic concentrations. The superior microbicidal activity of TauBr compared to TauCl was partially reversed under serum load (Table 4). This observation is in agreement with the recent report of Gottardi et al. (2014). However, in spite of some disadvantages TauBr seems to be a good candidate as an antiseptic in oral cavity infections and periodontal diseases. Thus, to prove this thesis, we have examined TauBr ability to kill *S. mutans* and *C. albicans* hidden in a biofilm generated *in vitro* on composite plates, the orthodontic material with the highest rate of microbial adhesion (Fig. 2).

3.3 Microbicidal Activity of TauBr and CHX Against S. mutans *and* C. albicans *Hidden in Biofilm (Grown on Composite Plates)*

In previous studies we have shown that TauBr is not able to destroy the structure of mature *P. aeruginosa* biofilm and to kill sessile bacteria, despite its strong bactericidal activity against planktonic form of *P. aeruginosa* (Marcinkiewicz et al. 2013b; "Taurine Haloamines and Biofilm. Part I: Antimicrobial Activity of Taurine Bromamine and Chlorhexidine Against Biofilm Forming *Pseudomonas aeruginosa*", Taurine 9 book). Such a lack of effect may be explained by a well-known resistance of biofilms to antibiotics and antiseptics. As such resistance depends on both partners, namely, bacteria and chemotherapeutics, it was rational to check

Table 5 The effect of long exposition of *S. mutans* hidden in biofilm to CHX and TauBr

	Streptococcus mutans (CFU/plate)	
	Time of incubation	
Tested compound	30 min	3 h
CHX	28 ± 8	4 ± 1
TauBr	>300	30 ± 4
CHX + TauBr	27 ± 3	7 ± 3
None (control)	>300	>300

Composite plates with 24 h biofilm of *S. mutans* were incubated with 1 % CHX or 0.06 % TauBr for 30 min and 3 h. The results show the number of surviving bacterial cells (CFU/plate). Means values ± SD of three independent experiments

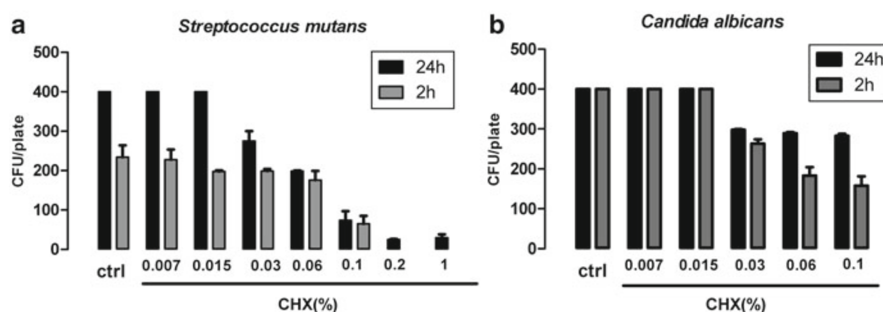

Fig. 3 Microbicidal activity of CHX and TauBr against *S. mutans* and *C. albicans* hidden in biofilm. Composite plates were incubated with the tested microbes for 2 or 24 h and then were exposed to CHX or TauBr for 30 min. The results show the number of surviving *S. mutans* (**a**) and *C. albicans* (**b**) cells isolated from the plates. Means values ± SD of three independent experiments. There was no effect of TauBr on the tested biofilms (data not shown)

anti-biofilm properties of TauBr against other bacterial species. The present results clearly indicate that TauBr used at micromolar concentrations and incubated with the mature *S. mutans* biofilm for a short time (30 min) is not able to kill sessile bacterial cells (data not shown). However, the number of viable bacteria (CFU/ml) was markedly reduced when the mature biofilm was exposed to TauBr for 3 h (Table 5). The effect of TauBr on *C. albicans* sessile cells was negligible under these circumstances. CHX showed strong anti-biofilm properties killing both tested microbes after short time of incubation. However, MIC of CHX against microbes hidden in a mature biofilm was much higher than that against planktonic cells (Fig. 3). These results are in keeping with other studies demonstrating excellent antimicrobial activity of CHX for most microorganisms tested in their free form, including *S. mutans*, but showing its lesser effectiveness against biofilm form (Bonez et al. 2013). On the other hand, also some disadvantages and adverse effects of CHX treatment in dentistry were reported. Yamaguchi et al. (2013) have shown that *Porphyromonas gingivalis* biofilms persist after CHX treatment. In addition, it has been reported that in endodontic therapy the generation of carcinogenic

para-chloroaniline is a potential consequence of the use of CHX in combination with NaOCl (Basrani et al. 2007; Rossi-Fedele et al. 2013). Only recently we have reported that NaOCl may be replaced by TauBr. Such combination of medicaments retains their anti-biofilm and antiseptic activities without stimulation of unwanted chemical reactions (Pasich et al. 2013a).

In summary, TauBr, as well as CHX, may be used as antiseptic and anti-inflammatory agents in dentistry, but its efficacy is related to its concentration and time of exposition. Therefore, such conditions might be achieved either by a frequent application or slow liberation of the agent. To use varnishes containing required antiseptic seems to be the most effective form for the professional application of CHX in preventing and treating periodontal diseases in the recent years (Puig-Silla et al. 2008). We suggest that varnishes containing TauBr or TauBr mixed with CHX will enhance effectiveness of local therapy of oral cavity infections associated with excessive dental plaque formation.

4 Conclusions

The results confirmed CHX exceptional potential as a primary antiseptic in dentistry, especially in prevention and treatment of dental carries, periodontal diseases and mouth candidiasis. Moreover, our study shows that TauBr may be used alternatively or in combination with CHX in killing of oral biofilm pathogens, due to its strong antibacterial and anti-inflammatory properties reported in a number of previous papers (Marcinkiewicz et al. 2005, 2006, 2008; Walczewska et al. 2013).

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Thiotaurine Modulates Human Neutrophil Activation

Elisabetta Capuozzo, Alessia Baseggio Conrado, and Mario Fontana

Abbreviations

fMLP	*N*-formyl-methionyl-leucyl-phenylalanine
HTAU	Hypotaurine
PMA	Phorbol 12-myristate 13-acetate
PKC	Protein kinase C
ROS	Reactive oxygen species
TAU	Taurine
TTAU	Thiotaurine

1 Introduction

Neutrophils are well recognized as one of the major players during acute inflammation. They are typically the first leukocytes to be recruited to an inflammatory site and can eliminate pathogens by multiple means. Two different microbicidal mechanisms occur within the neutrophils: the oxidative and the non oxidative systems. The oxygen-dependent mechanism acts through generation of reactive oxygen species (ROS), and the oxygen-independent mechanism acts through production of antimicrobial peptides and proteolytic enzymes. During inflammation, neutrophils are activated in response to several agonists generating superoxide anion and other ROS by NADPH oxidase-dependent mechanisms. This functional response, termed

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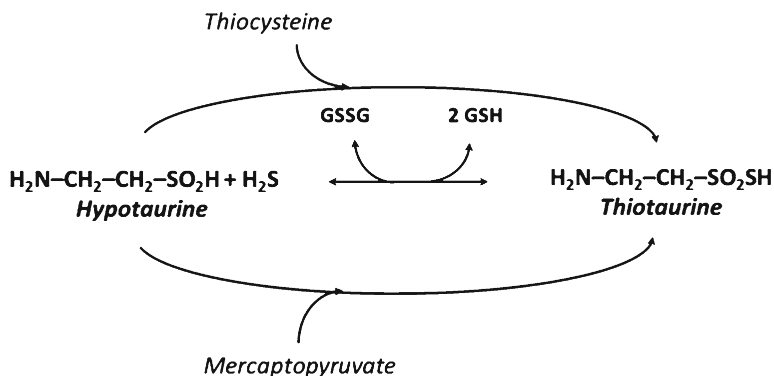
oxidative burst, contributes to host defense, but it can also result in collateral damage of host tissues. NADPH oxidase is a multicomponent enzyme system that catalyzes NADPH-dependent reduction of oxygen to superoxide anion. NADPH oxidase is activated by a variety of agents including *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) and the protein kinase C (PKC) activator phorbol 12-myristate 13-acetate (PMA). These stimuli trigger biochemical cascades leading to the phosphorylation of several proteins of the NADPH oxidase system (Morel et al. 1991). In addition to the well-documented PKC pathway, one of these cascades involves activation of members of the mitogen-activated protein kinase (MAPK) family. Several studies have demonstrated that MAPK pathways such as extracellular signal-regulated kinases (ERK) 1/2 and p38 MAPK are activated in human neutrophils (El Benna et al. 1996; Nick et al. 1997; Dewas et al. 2000).

Taurine is the most abundant free amino acid in most animal tissues and plays an important role in several essential biological processes (Huxtable 1992). A large number of reports have demonstrated the key role of taurine and its derivatives in the innate immune response (Schuller-Levis and Park 2004). It is widely recognized that taurine and related compounds such as hypotaurine and taurine chloramine exert a regulatory role in acute inflammation (Green et al. 1991; Marcinkiewicz and Kontny 2014; Kim and Cha 2014). The protection by taurine and its derivatives on inflammatory injury may be due to modulation of NADPH oxidase activity. It is noteworthy that taurine chloramines decrease PMA-stimulated superoxide production in human neutrophils by inhibiting phosphorylation of subunits of NADPH oxidase, eventually blocking the assembly of NADPH oxidase complex (Choi et al. 2006). Recently, it has been shown that thiotaurine (2-aminoethane thiosulfonate), a biomolecule structurally related to hypotaurine and taurine, prevents spontaneous apoptosis of human neutrophils (Capuozzo et al. 2013) and counteracts the damaging effect of oxidants in diabetic rat (Budhram et al. 2013). Interestingly, thiotaurine contains a sulfane sulfur that can be released as hydrogen sulfide (H_2S) (Westley and Heyse 1971; Capuozzo et al. 2013). It has been shown that H_2S plays relevant roles, modulating several pathophysiological processes, including inflammation (Zanardo et al. 2006; Predmore et al. 2012). Taken together, these observations raise the possibility that thiotaurine, analogously to taurine and its derivatives, could modulate neutrophil activation.

Thiotaurine is a thiosulfonates (RSO_2SH) which has been occasionally detected among the products of biochemical reactions involving sulfur compounds. Thiotaurine is a metabolic product of cysteine in vivo (Cavallini et al. 1959; Cavallini et al. 1960) and is produced by a spontaneous transsulfuration reaction involving thiocysteine (RSSH) and hypotaurine (RSO_2H) (De Marco et al. 1961) (Scheme 1). Moreover, a sulfurtransferase which catalyzes the transfer of sulfur from mercaptopyruvate to hypotaurine with production of thiotaurine has been also reported (Sörbo 1957; Chauncey and Westley 1983).

In the present study, thiotaurine has been assessed for an activity on functional response of human neutrophils. The results reveal that thiotaurine modulates fMLP- and PMA-mediated activation of human neutrophils, by inhibiting total ROS generation and superoxide anion production. Compared with fMLP-activated



Scheme 1 Biochemical pathway of thiotaurine

neutrophils, PMA-activated neutrophils were more susceptible to thiotaurine inhibition, suggesting that thiotaurine may interfere with the PKC-dependent pathway of neutrophil activation.

2 Materials and Methods

2.1 Chemicals

Thiotaurine (2-aminoethane thiosulfonate) was prepared from hypotaurine and elemental sulfur (Cavallini et al. 1959). Taurine, hypotaurine, sodium hydrosulfide (NaHS), sulfur, luminol, *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma-Aldrich, Inc (St. Louis, MO, USA). Cytochrome *c* from horse heart and cytochalasin B were from Fluka Chemie GmbH (Buchs, CH). MeOSuc-Ala-Ala-Pro-Val-AMC, elastase substrate, was from Enzo Life Sciences (Lausen, CH). All other chemicals were analytical grade.

2.2 Isolation of Neutrophils

Leukocytes were purified from heparinized human blood freshly drawn from healthy donors. Leukocyte preparations containing 90–98 % neutrophils were obtained by one-step procedure involving centrifugation of blood samples layered on Ficoll-Hypaque medium (Polymorphprep, Axis-Shield, Oslo, Norway) (Ferrante and Thong 1980). The cells were suspended in isotonic phosphate-buffered saline, pH 7.4, with 5 mM glucose and stored on ice. Each preparation produced cells with a viability higher than 90 % up to 6 h after purification. The incubations were carried out at 37 °C.

2.3 Respiratory Burst of Neutrophils: Luminol Enhanced Chemiluminescence

Human neutrophils were activated by 1 μM fMLP or by 1 μg/mL PMA. Total ROS production was evaluated by luminol enhanced chemiluminescence method (Klink et al. 2003). Neutrophils were distributed into 96-well black plate (1×10^6 cells/well) and incubated with test compound at various concentrations for 5 min at 37 °C. Then, PMA or fMLP was added to the cells to initiate respiratory burst and luminol (10 μM) to enhance chemiluminescence. Cytochalasin B (1 μg/mL) was added to fMLP-stimulated neutrophils. The chemiluminescence reading was recorded for 30 min at 2 min intervals. Chemiluminescence intensity was given in relative luminescence units (RLU). Data were expressed as the area under the curve of chemiluminescence (total RLU).

2.4 Respiratory Burst of Neutrophils: Superoxide Anion Production

Human neutrophils were activated by 1 μM fMLP or by 1 μg/mL PMA. Superoxide production by NADPH oxidase was estimated by measuring the rate of superoxide dismutase-inhibitable reduction of cytochrome *c* at 550 nm ($\varepsilon = 21{,}100\ M^{-1}cm^{-1}$ for ferrocytochrome *c*) by a modification of the method described by Lehmeyer et al. (1979). The incubation mixture contained 2×10^6 cells/mL, 80 μM cytochrome *c* in phosphate-buffered saline containing 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 5 mM glucose. After 3 min of preincubation at 37 °C, the reaction was started by adding PMA or fMLP. Cytochalasin B (1 μg/mL) was added to fMLP-stimulated neutrophils. The controls contained, in addition, 20 μg/mL superoxide dismutase. Steady-state velocity of superoxide production was estimated from the linear part of the reaction curve.

2.5 Determination of Neutrophil Degranulation by Elastase Release

Degranulation of azurophilic granules was determined by elastase release (Sklar et al. 1982). Elastase release was measured by hydrolysis of the elastase substrate (MeOSuc-Ala-Ala-Pro-Val-AMC). Briefly, isolated neutrophils (2×10^6 cells/mL) were resuspended in phosphate-buffered saline containing 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose and 1 μg/mL cytochalasin B at 37 °C. The elastase substrate (MeO-Suc-Ala-Ala-Pro-Val-AMC) was added at a final concentration of 40 μM. After 3 min of preincubation at 37 °C, the reaction was started by adding 1 μM fMLP, and elastase activity was monitored fluorometrically (excitation wavelength 380 nm, emission wavelength 460 nm).

2.6 Statistical Analysis

Results are expressed as mean ± SEM for at least three separate experiments. Graphics and data analysis were performed using GraphPAD prism 4 software. Statistical analyses were performed using the Student's *t*-test or the ANOVA and Bonferroni post hoc test. $p \leq 0.05$ was deemed significant.

3 Results

3.1 Effect of Thiotaurine and Related Compounds on Respiratory Burst of Human Neutrophils

The stimuli which trigger in vitro neutrophil response include substances, such as the chemotactic peptide fMLP, that bind to specific receptors, or substitutes of diacylglycerol, like PMA, that activate protein kinase C (PKC) directly. To investigate if thiotaurine could influence neutrophil response, thiotaurine was explored for its effect on respiratory burst. Preincubation of human neutrophil with 100 μM thiotaurine strongly reduced PMA-induced respiratory burst, as evaluated by luminol enhanced chemiluminescence (Fig. 1). Thiotaurine caused a decrease of respiratory burst of PMA-stimulated neutrophils in a dose-dependent manner (Fig. 1, inset). Addition of 100 μM thiotaurine induced 64.4 % inhibition of total ROS production by PMA-activated cells (Fig. 2). Conversely, fMLP-stimulated cells were slightly

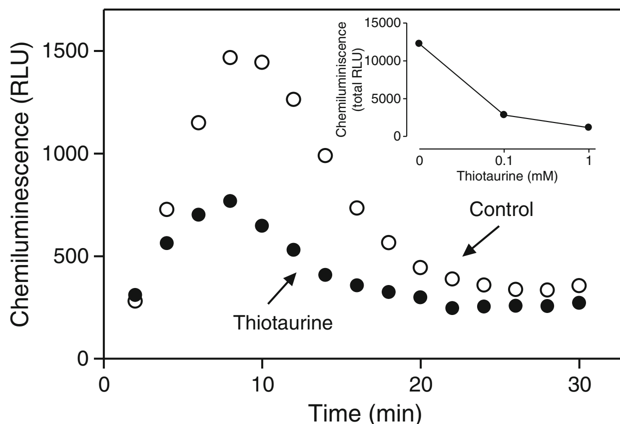


Fig. 1 Inhibition of human neutrophil respiratory burst in response to PMA by thiotaurine. Respiratory burst was evaluated by luminol enhanced chemiluminescence method. Neutrophils were incubated with 100 μM thiotaurine for 5 min at 37 °C. Then, 1 μg/mL PMA was added to initiate respiratory burst. The chemiluminescence reading was recorded for 30 min at 2 min intervals. Chemiluminescence intensity was given in relative luminescence units (RLU). Inset, dose-dependent inhibition of PMA-stimulated neutrophil respiratory burst by thiotaurine. Data were expressed as the area under the curve of chemiluminescence (total RLU)

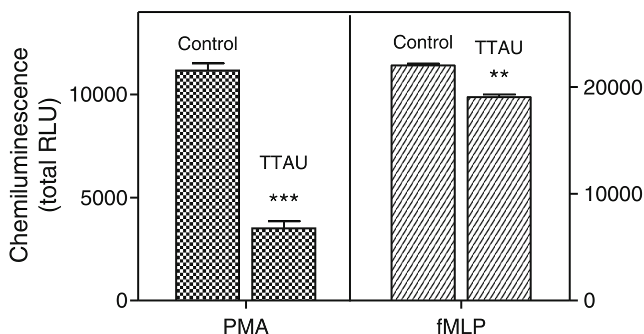


Fig. 2 Effect of thiotaurine on PMA- and fMLP-stimulated neutrophil respiratory burst. Respiratory burst was evaluated by luminol enhanced chemiluminescence method. Neutrophils were incubated with 100 μM thiotaurine for 5 min at 37 °C. Then, 1 μg/mL PMA or 1 μM fMLP was added to initiate respiratory burst. The chemiluminescence reading was recorded for 30 min at 2 min intervals. Data were expressed as the area under the curve of chemiluminescence (total RLU). $^{**}p<0.01$ and $^{***}p<0.001$, compared with the control value (PMA- or fMLP-stimulated neutrophil luminol enhanced chemiluminescence in the absence of thiotaurine)

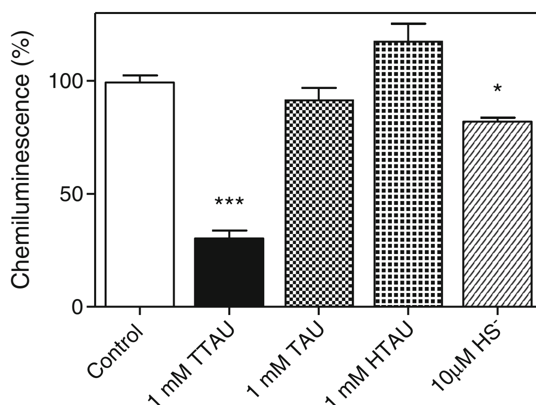


Fig. 3 Comparison of the effect of thiotaurine-related compounds and hydrogen sulfide on PMA-stimulated neutrophil respiratory burst. Respiratory burst was evaluated by luminol enhanced chemiluminescence method. Neutrophils were incubated at 37 °C. Thiotaurine and other test compounds at the indicated concentrations were added 5 min before activation by 1 μg/mL PMA. The chemiluminescence reading was recorded for 30 min at 2 min intervals. Data were estimated from the area under the curve of chemiluminescence and expressed as percentage of the control. $^{*}p<0.05$ and $^{***}p<0.001$, compared with the control value (PMA-stimulated neutrophil luminol enhanced chemiluminescence in the absence of thiotaurine)

affected by thiotaurine. A 13.4 % inhibition of respiratory burst was observed in fMLP-stimulated cells (Fig. 2).

As thiotaurine can release sulfane sulfur producing hydrogen sulfide and hypotaurine, the effect of these compounds on neutrophil respiratory burst has been evaluated. Thiotaurine (TTAU, 1 mM) was more effective than taurine (TAU, 1 mM), hypotaurine (HTAU, 1 mM) and hydrogen sulfide (HS^-, 10 μM) (Fig. 3).

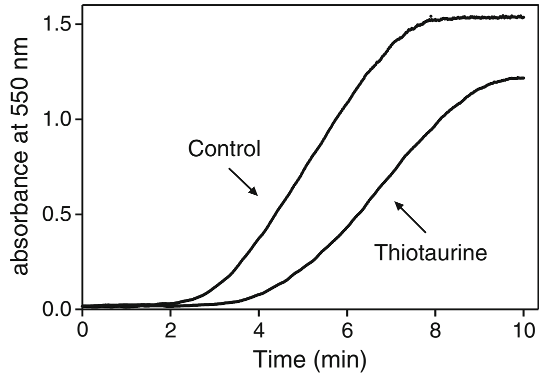


Fig. 4 Time course of superoxide anion generation by PMA-activated neutrophils with/without 1 mM thiotaurine. Superoxide anion generation was estimated by measuring cytochrome *c* reduction as described in Sect. 2. The absorbance at 550 nm was monitored. Thiotaurine was added 3 min before activation

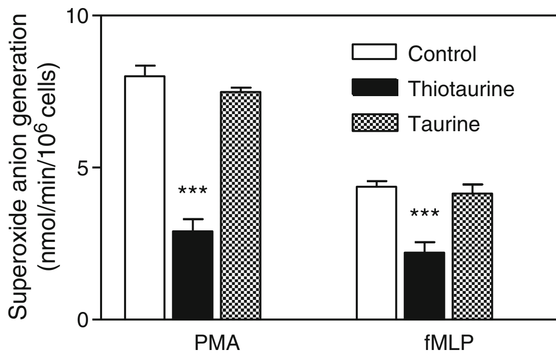


Fig. 5 Effect of thiotaurine and taurine on PMA- and fMLP-stimulated superoxide anion generation. Superoxide anion generation was estimated by measuring cytochrome *c* reduction as described in Sect. 2. Neutrophils were activated by 1 μg/mL PMA or 1 μM fMLP at 37 °C and the absorbance at 550 nm was monitored. Thiotaurine or taurine (1 mM) was added 3 min before activation. Steady-state velocity of superoxide production was estimated from the linear part of the reaction curve. ***$p<0.001$, compared with the control value (PMA- or fMLP-stimulated neutrophil superoxide anion generation in the absence of thiotaurine)

3.2 Thiotaurine Inhibition of Superoxide Anion Generation by Human Neutrophils

Superoxide anion is produced by neutrophils as a result of NADPH oxidase activation. NADPH oxidase has been activated by chemotactic peptide, fMLP or by PKC activator, PMA. Addition of 1 mM thiotaurine to human neutrophils in suspension did not stimulate superoxide anion generation. However, 1 mM thiotaurine added to neutrophils 3 min before activation by 1 μg/mL PMA led to an inhibition of superoxide anion generation, as estimated by measuring cytochrome *c* reduction (Fig. 4). Thiotaurine (1 mM) exhibited 58.6 and 49.4 % inhibition of superoxide anion generation by PMA- and fMLP-stimulated human neutrophils, respectively (Fig. 5).

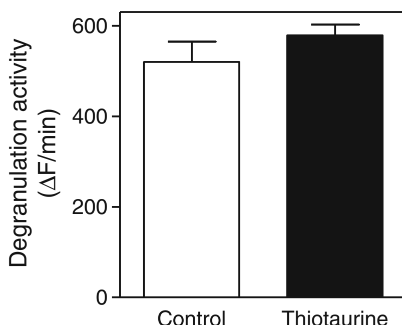


Fig. 6 Effect of thiotaurine on fMLP-stimulated neutrophil degranulation. Degranulation activity was determined by elastase release. Elastase activity was measured fluorometrically by hydrolysis of the elastase substrate (MeOSuc-Ala-Ala-Pro-Val-AMC) as described in Sect. 2. Neutrophils were incubated with 1 mM thiotaurine at 37 °C for 3 min before activation by 1 μM fMLP

3.3 Effect of Thiotaurine on Human Neutrophil Degranulation

Pretreatment of neutrophils with thiotaurine up to 5 mM concentration did not affect fMLP-induced azurophilic degranulation as evaluated by measuring the activity of released elastase (Fig. 6). Addition of thiotaurine to unstimulated neutrophils had no effect on their degranulation activity.

4 Discussion

Thiotaurine, a biomolecule structurally related to hypotaurine and taurine, has recently been found to prevent spontaneous apoptosis of human neutrophils (Capuozzo et al. 2013). In the present study, the influence of thiotaurine on human neutrophil functional responses was examined to further characterize its bioactivity toward proinflammatory leukocytes, which play a pivotal role in acute inflammation. Thiotaurine (0.1–1 mM) inhibited neutrophil activation in response to PMA or fMLP, as indicated by the inhibition of total ROS production, evaluated by luminol enhanced chemiluminescence, and of superoxide anion generation, estimated by measuring cytochrome *c* reduction. These results reveal that this thiosulfonate can attenuate leukocyte functions.

Important functional responses such as activation of NADPH oxidase leading directly to superoxide anion production likely occur through more than one signal transduction pathway, depending on the type of stimuli tested. In this respect, it has been reported that fMLP triggers both ERK 1/2-MAPK and PKC pathways to phosphorylate $p47^{phox}$ leading to NADPH oxidase activation, whereas PMA-induced phosphorylation of $p47^{phox}$ is mainly dependent on conventional PKC activation

(Dewas et al. 2000). Interestingly, PMA-activated neutrophils were considerably more susceptible to the inhibitory effects of thiotaurine than fMLP-activated cells. Thiotaurine strongly inhibits PMA-induced total ROS production in human neutrophils. Conversely, fMLP-induced response is influenced by this biomolecule at minor extent. Moreover, fMLP-induced neutrophil azurophilic degranulation is completely unaffected by thiotaurine. Taken together, these data suggest that thiotaurine modulates the activation pathways which trigger neutrophil response by interfering mainly with a PKC-dependent pathway.

The molecular mechanism by which thiotaurine modulates human leukocyte activation is difficult to explain taking into account only these results. Thiotaurine exhibits a peculiar activity on the neutrophil functional response compared to structurally related compounds. Under our experimental conditions, hypotaurine and taurine are not effective in modulating the activation of neutrophils. Although thiotaurine and related compounds are well-known free radical scavengers, the inhibition of respiratory burst of human neutrophils by thiotaurine could not be ascribed only to its antioxidant action (Aruoma et al. 1998; Fontana et al. 2004; Acharya and Lau-Cam 2013). However, thiotaurine contains a sulfane sulfur that can be released as hydrogen sulfide (H_2S), which plays regulatory roles in inflammation (Zanardo et al. 2006). In agreement, we found that H_2S inhibits respiratory burst as evaluated by luminol enhanced chemiluminescence. Moreover, sulfane sulfur moiety displays the unique ability to attach reversibly to other sulfur atoms (Toohey 1989). On the basis of this property, sulfane sulfur has been found to have regulatory effects in diverse biological systems (Beinert 2000; Mueller 2006). These functions include activation or inactivation of enzymes (Toohey 2011). It is possible that sulfane sulfur of thiotaurine can modulate by this mechanism enzymes involved in the signalling cascades leading to human neutrophil activation. In this regard, we suggested a role of thiotaurine as a biochemical intermediate in the transport, storage and release of sulfide in mammalian cells, such as neutrophils (Capuozzo et al. 2013). This hypothesis is further supported by the fact that hypotaurine, present in leukocytes at millimolar concentration (Learn et al. 1990), can readily incorporate H_2S formed during inflammation with production of thiotaurine (De Marco and Tentori 1961).

The cellular mechanism by which thiotaurine exerts a regulatory effect on neutrophil functional responses requires further study. However, these results indicate that thiotaurine displays a notable bioactive role that may provide new insights into the molecular inflammatory mechanisms and lead to the development of new therapeutic approaches for inflammation.

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Perinatal Taurine Exposure Alters Hematological and Chemical Properties of Blood in Adult Male Rats

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Abbreviations

C	Control
Cr	Plasma creatinine concentration
BW	Body weight
BUN	Blood urea nitrogen
HOCl	Hypochlorous acid
HW	Heart weight
IGF-1	Insulin-like growth factor 1
i.p.	Intraperitoneal
KW	Kidney weight
MMPs	Metalloproteases
TD	Perinatal taurine depletion
TS	Perinatal taurine supplementation

1 Introduction

Taurine (2-aminoethanesulfonic acid) is a sulfur-containing amino acid found in many tissues particularly brain, myocardium, liver, muscle, kidney, and blood cells (Huxtable 1992; Sturman 1993). Taurine content in the body is highest during early

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postnatal life and declines with advancing age. Taurine plays diverse physiological functions beginning at conception and continuing throughout life (Roysommuti and Wyss 2014). Perinatal taurine supplementation promotes prenatal and postnatal growth and development and protects against adult diseases. Epidemiological studies indicate an inverse relationship between the incidence of cardiovascular diseases and consumption of diets high in taurine, particularly fish (Yamori et al. 2010). Animal studies also support the relationship between taurine and cardiovascular disease. Treatment of adult animals with diets high in taurine decreases the rate of age-related organ damage, especially damage to the heart, blood vessels, brain, and kidneys. Hypertension in animal models can also be prevented or reduced by dietary taurine supplementation (Roysommuti and Wyss 2014).

Prenatal taurine deficiency induces low birth weights and a high risk of cardiovascular-related diseases, including coronary vascular disease, hypertension, diabetes mellitus, and renal dysfunction (Sturman 1993). In addition, these changes can be transferred to the next generation (Aerts and Van Assche 2002). Perinatal taurine depletion does not alter arterial pressure in adult male and female Sprague–Dawley rats, but high sugar intake significantly increases the arterial pressure only in males perinatally depleted of taurine (Roysommuti et al. 2009b; Thaeomor et al. 2010). Further, in these males, the combination of taurine depletion and a high sugar diet blunts baroreflex control of heart rate and renal nerve activity and increases sympathetic nerve activity. In female taurine-depleted rats treated with glucose in tap water since weaning, similar baroreflex effects occur (Thaeomor et al. 2010), the reflexes return to control levels following treatment with an angiotensin converting enzyme inhibitor captopril, but not following treatment with the estrogen receptor blocker tamoxifen (Roysommuti et al. 2013; Thaeomor et al. 2010). This suggests that the high sugar-related baroreceptor dysfunction in adult female rats that were perinatally depleted of taurine is linked to the renin-angiotensin system but not estrogen. Whether this effect also occurs in adult male rats has not been tested.

Adverse effects of perinatal excess taurine exposure have not been definitively demonstrated in humans, but taurine supplementation in late pregnant rats stimulates postnatal growth and induces obesity and insulin resistance in adult offspring (Hultman et al. 2007). Recently, El Idrissi and colleagues indicate that 0.05 % taurine in drinking water for 4 weeks significantly increases arterial pressure in female, but not male, Long-Evans rats (El et al. 2013). Previous experiments indicate that perinatal taurine supplementation also alters renal function and arterial pressure in adult rats (Roysommuti et al. 2009a; Roysommuti et al. 2010a; Roysommuti et al. 2010b). Nevertheless, these long-term effects do not appear to lead to severe abnormalities. In contrast to supplementation, perinatal taurine depletion can cause more severe disorders in both young and adult animals (Sturman 1993).

Inflammation and metabolic disorders have been reported to underlie many diseases observed in the elderly, particularly cardiovascular diseases (Aroor et al. 2013). Hyperlipidemia and hyperglycemia are commonly observed in metabolic syndrome that is reported to underlie several disorders in adults (Ferland and Eckel 2011). In taurine transporter knockout compared to wild-type mice, red blood cell and platelet counts are significantly lower and the packed cell volume is

significantly smaller in taut−/− mice than in taut+/+ mice, while the other erythrocyte parameters are similar. In contrast, white blood cell count is significantly higher in taut−/− mice than in taut+/+ mice (Lang et al. 2003). Although perinatal taurine imbalance seems not to alter blood sugar, glucose tolerance, electrolyte balance, and hematocrit in young adult (7–8 weeks old) rats, it can lead to mild insulin resistance (Roysommuti et al. 2013). Similar changes have not been tested in older rats. Thus, the present study is designed to elucidate the effect of perinatal taurine depletion and supplementation on hematological parameters, plasma electrolytes, and lipid profiles in adult male rats.

2 Methods

Sprague–Dawley rats were bred at the Northeastern Animal Center, Khon Kaen University and maintained at constant humidity (60 ± 5 %), temperature (24 ± 1 °C), and light cycle (06.00–18.00 h). Female Sprague–Dawley rats were fed normal rat chow with 3 % beta alanine (taurine depletion, TD), 3 % taurine (taurine supplementation, TS) or tap water alone (control, C) from conception to weaning. Male offspring were fed with the normal rat chow and tap water ad libitum throughout the experiment. At 13–16 weeks of age, the male rats were anesthetized with Nembutal (50 mg/kg, i.p.) and blood volumes (about 5.0 ml) were collected from abdominal aortas to measure non-fasting plasma sodium, plasma potassium, plasma calcium, blood glucose, blood urea nitrogen, plasma creatinine, complete blood count, and lipid profiles. Finally, all animals are euthanized with a high dose of anesthesia followed by removal of heart and kidney.

While blood glucose concentration was immediately measured by a glucometer (Accu-Check Advantage II, Roche, USA), other blood parameters were measured by the Srinagarind Hospital Laboratory Unit (Faculty of Medicine, Khon Kaen University). All experimental procedures were approved by the Khon Kaen University Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health guidelines.

All data are expressed as mean ± SEM and were statistically analyzed using one-way ANOVA and an appropriate post hoc Duncan's multiple range test with a significant criterion of p-value less than 0.05 (Statmost version 3.5, Dataxiom Software, USA).

3 Results

At 13–16 weeks of age, body weight, heart to body weight, and kidney to body weight were not significantly different among the three groups, but the heart weight of TS was slightly and significantly higher than that of TD group (Table 1). Compared to control and TD, TS rats displayed higher red blood cell count ($P < 0.05$), hemoglobin concentration ($P < 0.05$), and hematocrit ($P < 0.05$). Mean corpuscular

Table 1 Adult body, heart, and kidney weights in experimental groups

	Treatment		
	C (n = 18)	TD (n = 24)	TS (n = 14)
BW (g)	373 ± 7	350 ± 11	361 ± 14
HW (g)	1.20 ± 0.03	1.12 ± 0.02	1.21 ± 0.04[b]
KW (g)	2.47 ± 0.08	2.49 ± 0.06	2.53 ± 0.09
HW/BW (%)	0.32 ± 0.01	0.32 ± 0.00	0.34 ± 0.01
KW/BW (%)	0.66 ± 0.02	0.72 ± 0.02	0.71 ± 0.03

Data are mean ± SEM
C control, *TD* perinatal taurine depletion, *TS* perinatal taurine supplementation, *BW* body weight, *HW* heart weight, *KW* kidney weight
[b] $P < 0.05$ compared to TD

Table 2 Complete blood count (CBC) in experimental groups

	Treatment		
	C (n = 18)	TD (n = 20)	TS (n = 8)
Red blood cell (×10^3)/μl	6.9 ± 0.3	7.5 ± 0.2	8.2 ± 0.3[a]
Hematocrit (%)	40.0 ± 1.2	41.9 ± 0.9	46.1 ± 2.0[a,b]
Hemoglobin (g/dl)	13.5 ± 0.4	14.1 ± 0.3	15.5 ± 0.5[a,b]
Mean corpuscular volume (fl/cell)	58.4 ± 1.2	56.2 ± 0.1	56.4 ± 0.6
Mean corpuscular hemoglobin (pg/cell)	19.6 ± 0.3	19.0 ± 0.2	19.0 ± 0.3
Mean corpuscular hemoglobin concentration (g/dl)	33.6 ± 0.2	33.8 ± 0.4	33.8 ± 0.4
Red blood cell distribution width (%)	16.9 ± 1.2	14.2 ± 0.1	16.9 ± 1.4
White blood cell (×10^3)/μl	6.6 ± 1.3	4.4 ± 0.5	5.9 ± 1.0
Neutrophil (%)	34.1 ± 5.1	21.0 ± 3.2	18.8 ± 2.9[a]
Eosinophil (%)	0.9 ± 0.1	0.7 ± 0.2	1.3 ± 0.5
Basophil (%)	0.7 ± 0.2	1.2 ± 0.5	0.2 ± 0.1
Lymphocyte (%)	62.1 ± 5.3	74.5 ± 3.0	76.6 ± 3.6
Monocyte (%)	2.3 ± 1.0	2.3 ± 0.7	3.1 ± 1.2
Platelet (×10^3)/μl	979 ± 54	721 ± 46[a]	717 ± 70[a]
Mean platelet volume (fl)	5.4 ± 0.1	5.9 ± 0.1	6.5 ± 0.4[a,b]

Data are mean ± SEM
C control, *TD* perinatal taurine depletion, *TS* perinatal taurine supplementation
[a, b] $P < 0.05$ compared to C and TD, respectively

volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and red blood cell distribution width were not significantly different among groups (Table 2).

Perinatal taurine depletion and supplementation significantly decreased platelet count, whereas only the perinatal taurine supplementation (compared to control and TD) significantly increased mean platelet volume ($P < 0.05$). Although total white blood cells, lymphocytes, monocytes, eosinophils, and basophils were not significantly different among experimental groups, neutrophils were significantly lower in TS (45 % decrease) compared to control and TD groups ($P < 0.05$).

Table 3 Blood glucose and plasma electrolytes in experimental groups

	Treatment		
	C	TD	TS
Blood sugar (mg/dl)	141.4 ± 6.3 (n = 18)	146.5 ± 7 (n = 24)	166.2 ± 14.8 (n = 14)
Blood urea nitrogen (mg/dl)	21.8 ± 0.5 (n = 18)	24.1 ± 0.8 (n = 21)	18.9 ± 1.9[b] (n = 14)
Plasma creatinine (mg/dl)	0.31 ± 0.01 (n = 18)	0.30 ± 0.02 (n = 21)	0.30 ± 0.01 (n = 14)
BUN/Cr	70.5 ± 2.1 (n = 18)	84.0 ± 5.4 (n = 21)	65.0 ± 6.2[b] (n = 14)
Na^+ (mEq/l)	142.6 ± 0.7 (n = 18)	141.2 ± 0.5 (n = 21)	140.7 ± 1.2 (n = 13)
K^+ (mEq/l)	3.5 ± 0.1 (n = 15)	4.3 ± 0.2[a] (n = 18)	4.8 ± 0.3[a] (n = 8)
Ca^{2+} (mEq/l)	10.9 ± 0.1 (n = 15)	11.0 ± 0.1 (n = 18)	11.0 ± 0.5 (n = 8)

Data are mean ± SEM
C control, *TD* perinatal taurine depletion, *TS* perinatal taurine supplementation, *BUN* blood urea nitrogen, *Cr* plasma creatinine concentration
[a,b]$P < 0.05$ compared to C and TD, respectively

Table 4 Plasma lipid profiles in experimental groups

	Treatment		
	C (n = 18)	TD (n = 21)	TS (n = 14)
Cholesterol	71.1 ± 2.1	67.1 ± 2.7	73.9 ± 5.0
Triglyceride	53.3 ± 3.5	56.6 ± 3.5	59.5 ± 5.0
HDL	55.4 ± 2.7	53.5 ± 3.1	50.4 ± 3.5
LDL	17.0 ± 0.7	14.7 ± 0.5	21.7 ± 4.7[a]

Data are mean ± SEM
C control, *TD* perinatal taurine depletion, *TS* perinatal taurine supplementation
[a]$P < 0.05$ compared to TD

Non-fasting blood sugar, plasma sodium, and plasma calcium levels were not significantly different among the three groups, but both TD and TS (compared to control) displayed a similar increase in plasma potassium level (Table 3). Further, blood urea nitrogen, plasma creatinine concentration, and blood urea nitrogen to plasma creatinine ratio (BUN/Cr) were not significantly different in TD and TS, compared to control group. Despite being within a normal limit, TD displayed significantly higher blood urea nitrogen and BUN/Cr than the TS group.

Compared to controls, plasma lipid profiles were not altered by perinatal taurine depletion or supplementation; however, low density lipoprotein levels were significantly higher in TS compared to TD (but not compared to control) rats (Table 4).

4 Discussion

Ten-month-old, taurine transporter knockout mice display decreased red blood cells and platelets but increased white blood cells along with a reduction in plasma and red blood cell taurine content (Lang et al. 2003). This suggests that taurine deficiency leads to alteration in the balance of blood cells. However, the long-term

effects of perinatal taurine supplementation on hematological parameters have not previously been clarified.

The present study indicates that either depletion or supplementation of perinatal taurine alters hematological parameters in adult male rats. The taurine supplemented rats displayed increased red blood cell counts, hematocrit, and hemoglobin, but decreased neutrophils and platelets. The perinatal taurine supplementation did not affect red blood cell volume, hemoglobin concentration, or red blood cell distribution width, but it increased mean platelet volume. In comparison to supplementation, perinatal taurine depletion affected hematological parameters less, with only the platelet number but not volume being decreased in the adult male rats. Rats in the present study were perinatally depleted of, or supplemented with, taurine and fed with a normal taurine-containing diet after weaning. Thus, the present data suggest that perinatal taurine exposure alters hematological function in adults, despite their receipt of normal taurine during most of their life.

In 2010, Anand and colleagues reported that in normal adult Wistar rats, 60 days of oral taurine supplementation decreased platelet count, mean corpuscular hemoglobin, and percent lymphocytes, but increased percent neutrophils (Anand et al. 2010). However, neutrophil's phagocytic activity was significantly lowered by taurine supplementation. The similarity of the decrease in platelet count to the present findings suggests that the hematological effect of perinatal taurine supplementation might not be due to high taurine levels in the adult, but rather to a programming effect of taurine in early life. Measurement of plasma and blood cell taurine levels could help resolve the underlying mechanisms.

Several experiments indicate that blood cells, particularly platelets and neutrophils, are rich of taurine as are red blood cells to a lesser extent (Learn et al. 1990). Taurine possesses anti-platelet aggregation, anti-inflammatory, and anti-oxidant properties. In addition, taurine accelerates thrombolysis but increases red blood cell stability (Gossai and Lau-Cam 2009). In vitro, taurine at room temperature significantly enhances the stability of the platelet and red blood cell count, mean corpuscular hemoglobin, and red blood cell distribution width (Sirdah et al. 2013). Thus, the present finding of increased red blood cells, hematocrit, and hemoglobin in perinatal taurine supplemented rats might, at least in part, reflect enhanced stability of red blood cell.

Taurine reduces calcium influx evoked by aggregating agonists, thereby downregulating platelet aggregation (McCarty 2004b). Thus, compared to omnivores, vegans who are usually depleted of taurine are more sensitive to pro-aggregating agonists (McCarty 2004a). However, the effect of taurine on blood coagulation is complex and depends on doses of taurine, duration of treatment, and subject variability. Taurine supplementation decreases platelet aggregation in both normal and high fat-fed Sprague–Dawley rats (Park et al. 2007). While in rats, taurine supplementation decreases aggregation, in humans, the situation is more complex. In overweight pre-diabetic men, 1.5 g supplementation of taurine for 8 weeks does not affect platelet aggregation (Spohr et al. 2005), and in healthy Japanese people, there was no significant correlation between urine taurine concentration and global thrombosis test-occlusion times (measuring reactivity of platelets) (Ijiri et al. 2013).

In contrast, urine taurine concentration is inversely correlated with global thrombosis/lysis times (showing spontaneous thrombolytic activity).

Rupture of atherosclerotic plaque triggers the majority of myocardial infarctions. The rupture is activated by matrix metalloproteases (MMPs) secreted by intimal macrophages and foam cells (McCarty 2004c), and the activation of MMPs is mediated, in large part, by phagocyte-derived hypochlorous acid (HOCl). In vivo, the unrestrained oxidant activity of HOCl is opposed by taurine, which reacts spontaneously with HOCl to generate taurochloramine (Ogino et al. 2009). Taurochloramine possesses lesser oxidant activity than HOCl. Thus, taurine supplementation may be potentially useful to prevent sudden rupture of atherosclerotic plaque, while slowly increasing thrombolysis.

In the present study, perinatal taurine supplementation induced thrombocytopenia and increased mean platelet volume. Thrombocytopenia may occur due to an imbalance of platelet production and destruction; a larger platelet volume is commonly observed due to increased platelet production (Gladwin and Martin 1990; Threatte 1993). It is likely that the low platelet level in this study resulted from an increase in platelet destruction rather than a decreased production. This is supported by the observed rise in red blood cell count, hematocrit, normal red blood cell size, and normal hemoglobin content in adult TS rats. Thus, low plasma platelet concentration stimulates blood marrow to increase cell production leading to increases in new platelets and red blood cells. It does not appear that release of erythropoietin from the kidney had a major influence on the present results, since the kidney seems to function normally, as indicated by normal blood urea nitrogen, plasma creatinine, and blood urea nitrogen to plasma creatinine ratio.

Either prenatal taurine depletion or intrauterine growth restriction induces low birth weight and obesity, diabetes mellitus, and hypertension in adults (Roysommuti and Wyss 2014). In contrast, perinatal taurine depletion or supplementation does not induce obesity, diabetes mellitus, and hypertension in 7–8 weeks old Sprague–Dawley rats (Roysommuti et al. 2009c). However, mild insulin resistance is present in adult rats that were perinatally taurine depleted (but not in perinatal taurine supplemented rats) (Roysommuti et al. 2013). The present data further indicate that at 13–16 weeks old, neither TD nor TS rats develop obesity or diabetes mellitus. This differs from some previous reports, due perhaps to the fact that our rats were fed a normal taurine containing diet after weaning and throughout life. Malnourished mice fed a high fat diet develop obesity and glucose intolerance, and this is prevented by 5 % taurine supplementation after weaning (Batista et al. 2013). Epidemiologic studies also report that diets high in taurine can prevent or decrease obesity, diabetes mellitus, and hypertension in humans (Yamori et al. 2010).

Our previous experiments indicated that perinatal taurine depletion or supplementation do not affect plasma sodium or potassium levels in either male and female, 7–8 weeks old Sprague–Dawley rats (Roysommuti et al. 2009c). The present data indicate that in 13–16 weeks old rats, although perinatal taurine imbalance does not affect plasma sodium and calcium levels, it increases plasma potassium. In the 7–8 weeks old rats, prenatal and postnatal taurine depletion or supplementation has no effect on renal potassium excretion in males, but in females, perinatal taurine

depletion (but not supplementation) decreases urinary potassium excretion (Roysommuti et al. 2010b). Whether renal dysfunction underlies high plasma potassium levels in the present study is unclear; however, the normal blood urea nitrogen and plasma creatinine suggest that renal function was normal. The high blood cell turnover rates suggest that hyperkalemia due to increased hemolysis was present in TS rats; this is not to the case in TD rats which displayed normal hematological parameters.

Taurine supplementation has been shown to decrease hyperlipidemia in many animal models and humans (Chen et al. 2012; Yamori et al. 2010). However, other studies show that 2.5 % taurine supplementation fails to improve hyperlipidemia and endothelial function, but reduces apoptosis and atherosclerosis in high fat fed rabbits (Zulli et al. 2009). Differences in doses, duration of treatment, and animal models may underlie the efficacy of taurine supplementation. Maternal taurine supplementation in late-pregnant rats markedly stimulates postnatal growth in the offspring (Hultman et al. 2007). In 12-week-old females, fat deposits and plasma free fatty acid increased without any effect on plasma cholesterol, triglycerides or serum IGF-1, while in males, only fat deposits increase, consistent with the present findings.

5 Conclusion

In the rat, perinatal taurine exposure alters adult hematological function. The present study indicates that both perinatal taurine supplementation and depletion can adversely alter hematologic parameters in adult rats. High taurine diets are recommended for women during pregnancy and lactation to prevent potential short- and long-term effects of taurine deficiency. The present results suggest that caution and further studies may be warranted, since perinatal taurine supplementation has potentially adverse effects on adults, at least in rats.

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Effect of Taurine on Viability and Proliferation of Murine Melanoma B16F10 Cells

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1 Introduction

Apoptosis is the process of programmed cell death and dysregulated apoptosis is involved in a variety of diseases such as cancer and neurodegenerative diseases. Agents that suppress the proliferation of malignant cells, and even cause apoptosis, have the potential to both prevent and treat cancer (Parra et al. 2011). Taurine (2-aminoethanesulfonic acid) is a sulfur-containing β-amino acid that is present widely in mammals. It is one of the end-products of cysteine metabolism in mammals and is renally excreted. Taurine exhibits pharmacological actions and various

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beneficial physiological functions. These benefits have encouraged the consumption of seafood containing high concentrations of taurine and the use of taurine in infant formulas, nutritional supplements, and energy drinks (Matsuda and Asano 2012). Moreover, taurine has significant anti-inflammatory properties (Marcinkiewicz 2009) and participates in different physiological processes as it stabilizes cell membranes (Condron et al. 2010) and regulates fatty tissue metabolism (Ueki and Stipanuk 2009) and levels of calcium ions in blood (Ribeiro et al. 2010). The nonmetabolizable β-amino acid taurine suppresses inflammation (Marcinkiewicz and Kontny 2014) and reduces hepatic lipid oxidative stress (Balkan et al. 2002), protecting liver function during ethanol metabolism (Kerai et al. 1998; Yang et al. 2010). Because it is nonmetabolizable, the protective mechanism of taurine is not yet known, but its anti-inflammatory and antioxidative activities (Marcinkiewicz and Kontny 2014) primarily result from its sequestration of HOCl and HOBr (Weiss et al. 1982). Taurine has a protective effect in murine hepatocytes against oxidative stress-induced apoptosis by tert-butyl hydroperoxide (Roy and Sil 2012), exerts a hypoglycemic effect, and suppresses mitochondria-dependent apoptosis in renal and cardiac tissues of alloxan-induced diabetic rats (Das and Sil 2012; Das et al. 2012). There is an increasing interest in studying the additive or synergistic effect of taurine in various diseases including cancer. However, the anticancer activity of taurine in melanoma cells has not been sufficiently studied. In this study, it was determined whether taurine exhibits an anticancer activity that targets proliferation and apoptosis of B16F10 cells.

2 Materials and Methods

2.1 Materials

Taurine, MTT, neutral red, and propidium iodide (PI) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Antibodies were purchased from Santa Cruz Biotechnology (Delaware, CA, USA). β-actin antibody was purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). All other reagents were of the highest grade commercially available.

2.2 Cell Culture

The murine melanoma B16F10 cell line was obtained from the American Type Culture Collection (Rockville, MD) and maintained in Dulbeco's Modified Eagle's Medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10 % heat-inactivated fetal bovine serum (FBS) (Gibco), penicillin (100 U/mL), and streptomycin (100 μg/mL) under 5 % CO_2 in a humidified incubator at 37 °C.

2.3 MTT Assay

The anti-proliferation assay was performed according to a well-established MTT method with slight modifications (Carmichael et al. 1989). Briefly, B16F10 cells (2.0×10^4 cells/well) were seeded in 96-well culture plates. Cells were treated with samples for 24 h, MTT solution was added into each well, and cells were incubated for 3 h. The medium was discarded and the intracellular formazan product dissolved in 150 μL dimethyl sulfoxide (DMSO) under continuous shaking for 10 min. The absorbance was measured at 540 nm using a microplate reader (Tecan, Austria). Cell viability was expressed as a percentage of the control.

2.4 Neutral Red Assay

The neutral red assay is based on the incorporation of neutral red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) into the lysosomes of viable cells. Briefly, B16F10 cells (2.0×10^4 cells/well) were seeded in 96-well culture plates. Cells were treated with taurine (5, 10, and 20 mM) for 24 h, 200 μL of prepared neutral red solution was added to each well, and the cells were incubated at 37 °C for 24 h. Subsequently, the cells were rapidly washed with a solution of 0.1 % calcium chloride and 0.5 % formaldehyde. To extract the dye from the intact and viable cell, a solubilization solution of 1 % acetic acid and 50 % ethanol was added to the cells. Following 10 min incubation at room temperature, the absorbance (OD) was measured by spectrophotometry at 540 nm. Results were expressed as percentages of the control.

2.5 Cell Cycle Analysis

Cellular DNA content was measured using flow cytometry. Briefly, the cells (1.0×10^5) were seeded in 6-well plates and allowed to adhere overnight. Cells were treated with various concentrations of taurine (5, 10, and 20 mM) for 24 h. The cells were harvested by trypsin treatment, washed with cold phosphate-buffered saline (PBS, pH 7.4), and stained with PI solution (50 μg/mL of propidium iodide, 10 μg/mL RNase, and 0.5 % Tween-20 in PBS). Cell cycle phase distribution and DNA histograms of the stained cells were determined by flow cytometry (FACSCalibur, BD Bioscience). Data from 1.0×10^3 cells per sample were collected and analyzed with CellQuest software (Becton Dickinson).

2.6 Determination of Morphologic Changes

B16F10 cells were seeded in 6-well plates (1.0×10^5 cells/well) and incubated in DMEM at 37 °C under 5 % CO_2 for 24 h. Following a 24 h incubation with taurine (5, 10, and 20 mM), cellular morphology was assessed using a phase-contrast microscope (Nikon, Japan). Images were taken at 200× magnification.

2.7 Hoechst 33342 Staining

B16F10 cells were plated in 6-well plates and treated with taurine (5, 10, and 20 mM) for 24 h, fixed in PBS containing 4 % formaldehyde for 30 min at room temperature. Fixed cells were washed with PBS containing 0.02 % Tween-20 and stained with 1 μg/mL Hoechst 33342 (Sigma, St. Louis, MO, USA) for 20 min at room temperature. The cells were subsequently washed twice with PBS and visualized and photographed using a fluorescence microscope.

2.8 Western Blot Analysis

Equal amounts of total protein (20 μg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Separated proteins were transferred electrophoretically onto polyvinylidene difluoride membranes (PVDF) and blocked with 5 % skimmed milk in TBS-T for 2 h. Blots were incubated with specific primary antibodies and the immune complexes detected using appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies. Incubation with the secondary antibody was followed by triplicate washes with TBS-T and the blots were processed for visualization using an enhanced-chemiluminescence (ECL) detection kit and a Luminescent Image Analyzer (LAS-3000, Fujifilm, Tokyo, Japan).

2.9 Statistical Analysis

The data are expressed as the mean ± standard deviation (SD). Statistical analyses were assessed by Student's *t*-test for paired data and one-way analysis of variance (ANOVA) followed by Duncan's post-hoc multiple range test. Graph Pad Prism software version 4.00 (Graph Pad Software Inc., San Diego, CA) was used. Results were considered significant at $p < 0.05$.

3 Results

3.1 Effect of Taurine on Cell Viability of B16F10 Cells

B16F10 cells were treated with various concentrations of taurine (5, 10, and 20 mM) and the percentage of surviving cells was assessed using both a MTT and neutral red assay. After treatment with taurine, cell viability of B16F10 cells was

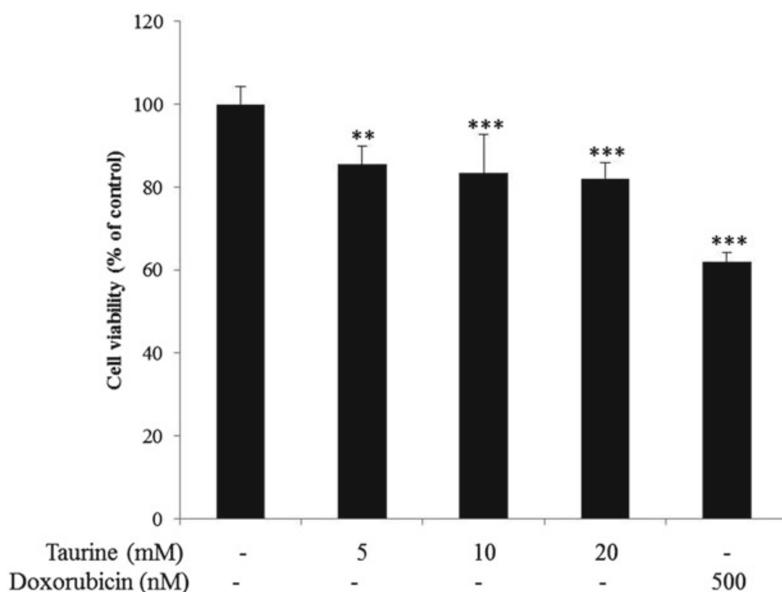

Fig. 1 Effect of taurine on cell viability in the MTT assay. B16F10 cells were treated with taurine for 24 h. Results are expressed as mean ± SD from three independent experiments. One-way ANOVA was used for comparisons of multiple group means followed by Dunnett's *t*-test (***$p < 0.001$, **$p < 0.01$ compared with control)

significantly decreased (Fig. 1). Cell viability upon treatment with 5, 10, and 20 mM of taurine was 85.4, 83.3, and 81.9 %, respectively as measured using the MTT assay and 95.9, 92.3, and 84.1 %, respectively as determined with neutral red detection. Treatment of cells with doxorubicin (500 nM) was used as a positive control with cell viability found to be 61.9 and 57.4 % for the MTT and neutral red assay, respectively. Both the MTT and neutral red assay showed that taurine treatment resulted in decreased cell viability in a dose-dependent manner (Fig. 2).

3.2 *Effects of Taurine on the Cell Cycle Distribution of B16F10 Cells*

Table 1 shows the representative histograms of the relative percentage of B16F10 cells in each phase of the cell cycle after incubation in the absence and presence (5, 10, and 20 mM) of taurine for 24 h. As determined by PI staining, the sub-G1 peak of B16F10 cells increased in a dose-dependent manner. Apoptosis was induced with 5.48, 3.90, and 1.66 % after treatment with 5, 10, and 20 mM taurine, respectively (Fig. 3).

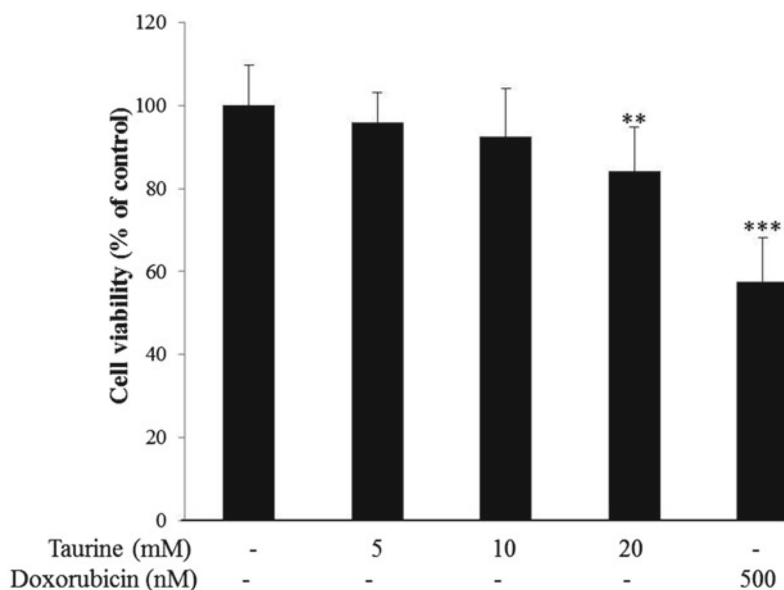

Fig. 2 Effect of taurine on cell viability in the neutral red assay. Results are expressed as mean ± SD from three independent experiments. One-way ANOVA was used for comparisons of multiple group means followed by Dunnett's *t*-test (***$p < 0.001$, **$p < 0.01$ compared with control)

Table 1 Effects of taurine on the cell cycle distribution of B16F10 cells

Group	Number of cell (%)			
	Sub G1	G1	S	G2/M
Control	1.47 ± 0.21	52.69 ± 2.52	8.85 ± 1.02	28.91 ± 2.13
Taurine (5 mM)	1.66 ± 0.12	51.98 ± 2.29	10.49 ± 1.13	28.90 ± 2.25
Taurine (10 mM)	3.90 ± 0.24	50.11 ± 3.26	10.24 ± 1.05	27.94 ± 2.19
Taurine (20 mM)	5.48 ± 0.15	45.73 ± 2.11	10.24 ± 1.23	28.68 ± 2.31
Doxorubicin (500 nM)	25.51 ± 2.22	30.04 ± 1.58	10.49 ± 1.21	28.64 ± 2.17

Cells were treated with the indicated concentration of samples for 24 h and stained with PI for flow cytometry analysis. The percentages of cells in each phase of three independent experiments are given

3.3 Apoptotic Effects and Morphological Changes in B16F10 Cells

Morphological changes and cell death in B16F10 cells were observed using an inverted microscope. The morphological changes observed after 24 h exposure to increasing concentrations of taurine are shown in Fig. 4. Control cells were not affected in their proliferation and showed normal cell morphology. However, treatment with taurine caused cell death and decreased cell density. In addition, changes in nuclear morphology were detected by the Hoechst 33342 nuclear staining.

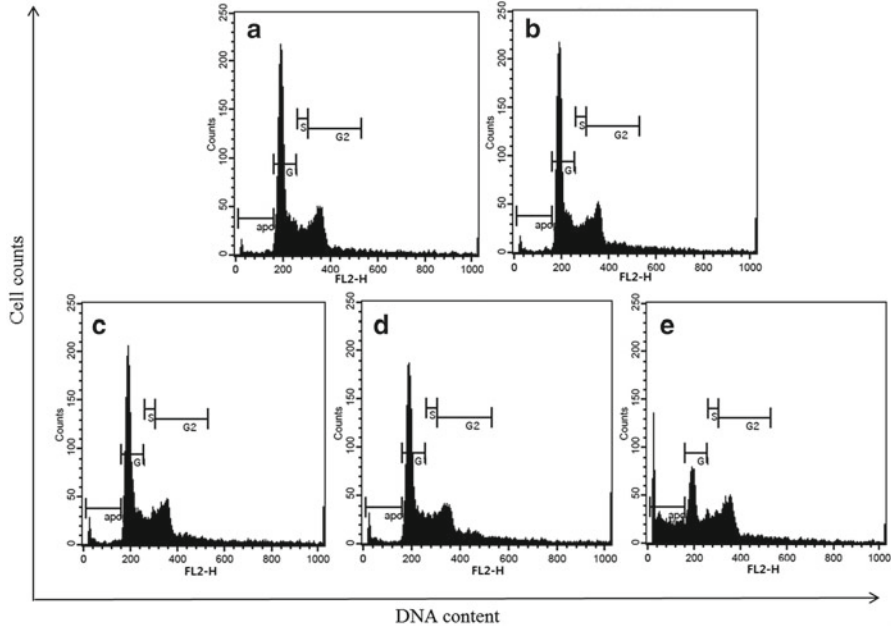

Fig. 3 Cell cycle analysis by flow cytometry. (**a**) Control, (**b**) 5 mM taurine, (**c**) 10 mM taurine, (**d**) 20 mM taurine, (**e**) 500 nM doxorubicin

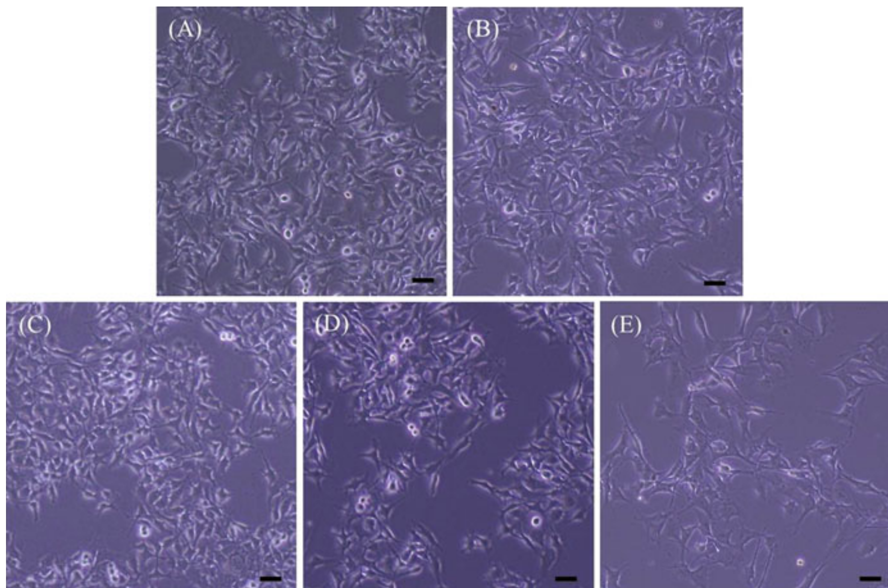

Fig. 4 Morphological changes in B16F10 cells. Cells were incubated with samples for 24 h and photographs were taken using an inverted microscope. Scale bar: 10 μm. (**a**) control, (**b**) 5 mM taurine, (**c**) 10 mM taurine, (**d**) 20 mM taurine, (**e**) 500 nM doxorubicin

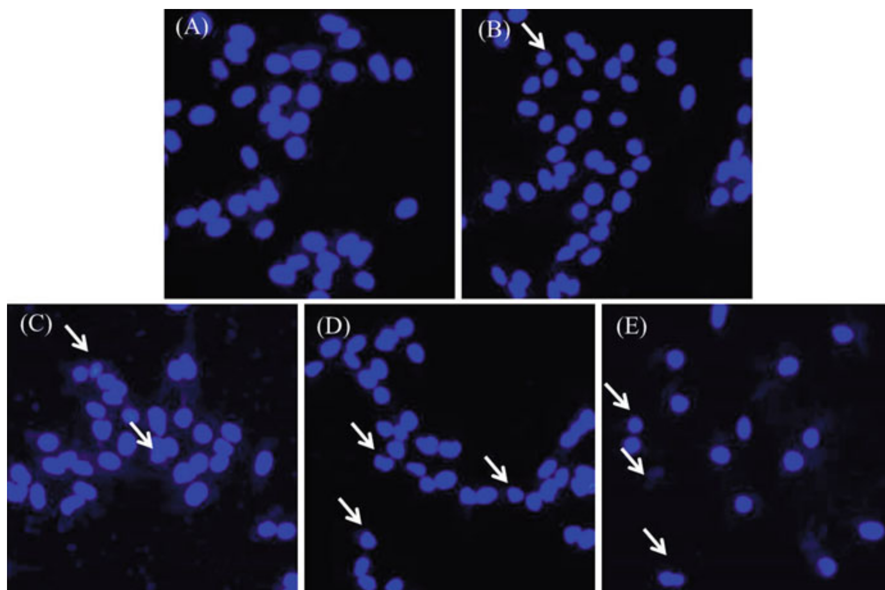

Fig. 5 Nuclear morphological changes in B16F10 cells. Cells were incubated with samples for 24 h, fixed with 4 % paraformaldehyde, and stained with Hoechst 33342. Photographs were taken using a fluorescent microscope (200× magnification). (**a**) Control, (**b**) 5 mM taurine, (**c**) 10 mM taurine, (**d**) 20 mM taurine, (**e**) 500 nM doxorubicin

As shown in Fig. 5, the nuclei in the control group were stained as weak homogenous blue, while bright chromatin condensation and nuclear fragmentation were observed in the taurine-treated groups.

3.4 Western Blot Analysis

To confirm apoptosis in response to taurine treatment, the expression levels of Bcl-2 and Bax, an antiapoptotic and proapoptotic protein, respectively, were evaluated by western blot analysis. The expression level of Bcl-2 decreased gradually with increasing taurine concentrations. In contrast, that of Bax did not change (Fig. 6).

4 Discussion

As with many cancers, the development of melanoma is associated with immune suppression (D'Agostini et al. 2005). The capacity to elicit effective T- and B-cell immunity, including anti-tumor cell activity, is ultimately related to stimulation of lymphocyte proliferation and the upregulation of the activity of both NK and CTL cells (Marciani et al. 2000; Zhang et al. 2005). Taurine, one of the most abundant

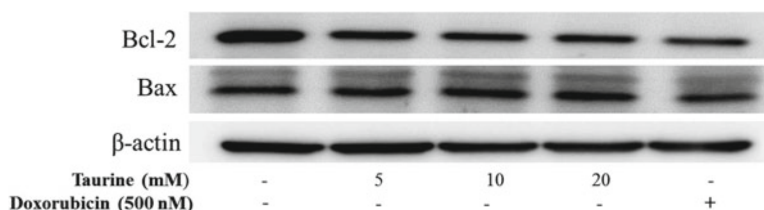

Fig. 6 Effect of taurine on the Bcl-2 family of proteins in B16F10 cells. Cells were treated with the indicated concentration of taurine for 24 h. Cells were subsequently lysed and equal amounts of cell protein (20 μg) were separated on SDS-polyacrylamide gels and transferred to PVDF membranes. The membranes were probed with the indicated antibodies. An ECL detection system was used for visualization of the proteins. β-actin was used as an internal control

free amino acids presents in mammalian tissues, regulates many cellular functions including inflammatory processes (Choi et al. 2006). Beside metabolic regulation, taurine also plays an important role in innate immunity (Nagl et al. 2000) and is directly related to antioxidant properties in clinical (Zulli 2011; Shivananjappa and Muralidhara 2012), toxicological (Turna et al. 2011; Yildirim and Kilic 2011; Shao et al. 2012), and oncological studies (Henderson et al. 2001; Gottardi and Nagl 2010; Shalby et al. 2011). Apoptosis is a regulated process involving changes in the expression of distinct genes. The Bcl-2 family of proteins (e.g., Bcl-2 and Bcl-xL) is a regulator of the apoptotic pathway. Bcl-2 and Bcl-xL are upstream molecules in this pathway and potent suppressors of apoptosis (Hockenbery et al. 1993). These Bcl-2 family genes mainly act in the mitochondrion and are involved in the survival/death pathway, where Bcl-xL and Bcl-2 are responsible for survival and Bak, Bax, and Bad for apoptosis (Basu and Haldar 1998). In this study, we found that taurine inhibits the proliferation of murine melanoma B16F10 cells via apoptosis. Taurine can block melanoma cell proliferation and induce apoptosis through a mitochondrial pathway. In summary, the antiproliferative effect of taurine on B16F10 melanoma cells was investigated. Cell viability was studied using the MTT and neutral red uptake test assays. Moreover, cell cycle analysis and protein expression upon taurine treatment were examined using western blotting and flow cytometry, respectively. These results indicate that taurine suppresses the proliferation of murine melanoma B16F10 cells.

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Investigation of Antioxidant and Anticancer Potential of Taurine by Means of Multiple Chemical and Biological Assays

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Abbreviations

ABTS	3-Ethyl-benzothiazoline-6-sulfonic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
FRAP	Ferric reducing antioxidant power
ORAC	Oxygen radical absorbance capacity
ROS	Reactive oxygen species
TE	Trolox equivalent

1 Introduction

Oxidative stress plays a crucial role in a lot of disease outbreaks and progression (Jacob and Burri 1996). Damage from ROS and free radicals has been linked to cancers (Goodwin and Brodwick 1995) and neurodegenerative disorders (Youdim and

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Joseph 2001). Moreover, oxidation of low-density lipoprotein is also an important factor in the promotion of coronary heart disease and atherosclerosis (Frankel et al. 1993). The importance of ROS and free radicals has attracted increasing attention over the past decade. ROS, which include free radicals such as superoxide anion radicals ($O_2{\bullet}^-$), hydroxyl radicals (OH•) and non-free-radical species such as H_2O_2 and singled oxygen ($^1O_2$), are various forms of activated oxygen. These molecules are exacerbating factors in cancer and aging process (Halliwell and Gutteridge 1989; Gulcin et al. 2002a, b). Therefore, research for identifying antioxidant compounds is an important issue. Antioxidants are substances that delay or prevent the oxidation of cellular oxidizable substrates. The synthetic antioxidants such as butylated hydroxyanisol (BHA), butylated hydroxyltoluene (BHT), tert-butylhydroquinone (TBHQ) and propyl gallate (PG) are added to food during processing to suppress lipid peroxidation and consequently to improve food quality and stability. However the use of these antioxidants in food products is under strict regulation due to the potential health hazards caused by such compounds (Cashman 1997). Therefore much more attention of investigators and consumers is paid to natural antioxidants for use in foods or medicinal materials to replace synthetic antioxidants.

Natural antioxidants have gained increasing interest among consumers and the scientific community because epidemiological studies have indicated that frequent consumption of natural antioxidants is associated with a lower risk of cancer and cardiovascular disease (Renaud et al. 1998; Temple 2000).

Several analyses have been frequently used to estimate antioxidant activities such as ORAC (Cao et al. 1993; Ou et al. 2001; Prior et al. 2003), DPPH (Brand-Williams et al. 1995; Gil et al. 2002), FRAP (Benzie and Strain 1999; Guo et al. 2003; Jimenez-Escrig et al. 2001), and ABTS (Leong and Shui 2002; Miller and Rice-Evans 1997). The ORAC assay is said to be more reliable because it utilizes a biologically relevant radical source (Prior et al. 2003). These techniques have shown different results among crop species and across laboratories. Ou et al. (2002) reported no correlation of antioxidant activity between the FRAP and ORAC techniques among most of the 927 freeze-dried vegetable samples, whereas these methods revealed high correlation in blueberry fruits (Connor et al. 2002). Similarly, Awika et al. (2003) observed high correlation between ABTS, DPPH, and ORAC among sorghum and its products. The aim of this research was to compare the efficiency of ORAC, DPPH, FRAP, and ABTS assays to estimate antioxidant activities and determine their anti-breast cancer activity in taurine.

2 Methods

2.1 Materials

Taurine, fluorescein sodium salt, DPPH, trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), which is a hydrophilic analogue of vitamin E were purchased from Sigma (St. Louis, MO, USA). All other reagents were of the highest grade available commercially.

2.2 Cell Culture

The human breast cancer cell lines, MCF-7 and MDA-MB-231, were cultured in DMEM medium, supplemented with 10 % FBS, 1 % penicillin/streptomycin in a 5 % CO_2 atm at 37 °C. The cells were seeded at a density of 3.5×10^5 cells well in a 13 cm well culture dish. After 24 h, the cells were treated with 0.125, 0.250, 0.500, and 1.000 mM of taurine in medium. Cells were treated with taurine for 24 h and then harvested.

2.3 ORAC Assay

For ORAC procedure, the method of Ou et al. (2001) was used with some slight modification using an automated plate reader (KC4, Bio Tek, USA) with 96-well plate. Analyses were conducted in phosphate buffer pH 7.4 at 37 °C. Peroxyl radical was generated using 2,2′-azobis (2-amidino-propane) dihydrochloride which was prepared fresh for each run. Fluorescein was used as the substrate. The fluorescence was measured immediately after the AAPH addition and measurements were taken every 5 min. Fluorescence conditions were as follows: excitation at 485 nm and emission at 520 nm. The standard curve was linear between 0 and 62.5 μM Trolox. Results were expressed as μM TE. The activity was calculated using the following equation:

$$\mathrm{ORAC}(\mu\mathrm{M\,TE}) = (\mathrm{C}_{\mathrm{Trolox}} \times (\mathrm{AUC}_{\mathrm{sample}} - \mathrm{AUC}_{\mathrm{blank}}) \times \mathrm{k}) / (\mathrm{AUC}_{\mathrm{sample}} - \mathrm{AUC}_{\mathrm{blank}})$$

$$\mathrm{AUC} = (0.5 + \mathrm{f}_5/\mathrm{f}_0 + \mathrm{f}_{10}/\mathrm{f}_0 + \ldots + \mathrm{f}_{n+5}/\mathrm{f}_0) \times 5$$

2.4 DPPH Radical Scavenging Activity

DPPH scavenging activity of various antler extracts was measured according to a slightly modified method of Brand-Williams et al. (1995). DPPH solution (1.5×10^{-4} M, 100 μL) was mixed with and without each extract (100 μL), after which the mixture was incubated at room temperature for 30 min. After standing for 30 min, absorbance was recorded at 540 nm by microplate reader and the standard curve was linear between 0 and 62.5 μM Trolox. Results were expressed as μM TE.

2.5 FRAP Assay

FRAP assay was done according to Benzie and Strain (1999). The fresh working solution was prepared by mixing acetate buffer, TPTZ solution and $FeCl_3 \cdot 6H_2O$ solution and then warmed at 37 °C before using. Each extract was allowed to react

with FRAP solution at dark room conditions and room temperature for 30 min. Readings of the colored product were then taken at 595 nm and the standard curve was linear between 0 and 62.5 μM Trolox. Results were expressed as μM TE.

2.6 ABTS Assay

The procedure of ABTS scavenging activity followed the method of Arnao et al. (2001). Stock solutions included ABTS•+ solution and potassium persulfate solutions. Working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h. The solution was then diluted with fresh ABTS•+ solution and mixed with or without each extract. After incubation for 2 h, the absorbance was recorded at 735 nm, and the standard curve was linear between 0 and 62.5 μM Trolox. Results were expressed as μM TE.

2.7 Cell Viability

Cell viability was determined by 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay in 96-well plates as previously described. Cells were incubated with various concentrations of taurine for 48 h followed by MTT for 4 h, and then 100 μL of isopropanol (in 0.04 N-hydrochloric acid) was added to dissolve the formazan crystals. The absorbance was read at 570 nm using the Anthos 2010 spectrophotometer (Salzburg, Austria). Cell viability was calculated as being the relative absorbance compared to absorbance compared to control (Kim et al. 2002).

3 Results

3.1 ORAC of Taurine

Means of the ORAC levels from taurine were 19.64, 24.43, 27.74, and 29.97 μM TE at 125–1,000 μg/mL (Table 1) (Fig. 1).

3.2 DPPH Radical Scavenging Activity of Taurine

Antioxidant activity was measured in taurine using DPPH assay, and the activity was 0.17, 1.13, 12.08, and 15.15 μM TE at 125–1,000 μg/mL (Table 2) (Fig. 2).

Table 1 Antioxidant activity of taurine by ORAC

Concentrations (μg/mL)	μM TE
125	19.64 ± 0.17
250	24.43 ± 0.23
500	27.74 ± 0.20
1,000	29.97 ± 0.08

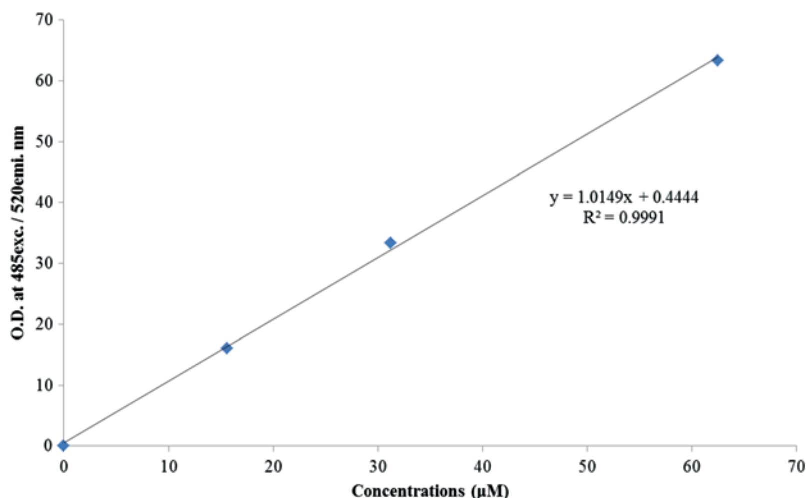

Fig. 1 Calibration curve for ORAC assay probe as a function of standard trolox concentrations

Table 2 Antioxidant activity of taurine by DPPH radical

Concentrations (μg/mL)	μM TE
125	0.17 ± 0.18
250	1.13 ± 0.23
500	12.08 ± 0.47
1,000	15.15 ± 0.77

3.3 *FRAP Assay of Taurine*

To verify the antioxidant capacity of taurine, we also evaluated FRAP from taurine. The activity was 6.34, 8.61, 9.49, and 17.51 μM TE at 125–1,000 μg/mL (Table 3) (Fig. 3).

3.4 *ABTS Assay of Taurine*

The TE values were 6.18, 29.16, 45.13, and 52.09 μM as determined by the ABTS assay at 125–1,000 μg/mL (Table 4) (Fig. 4).

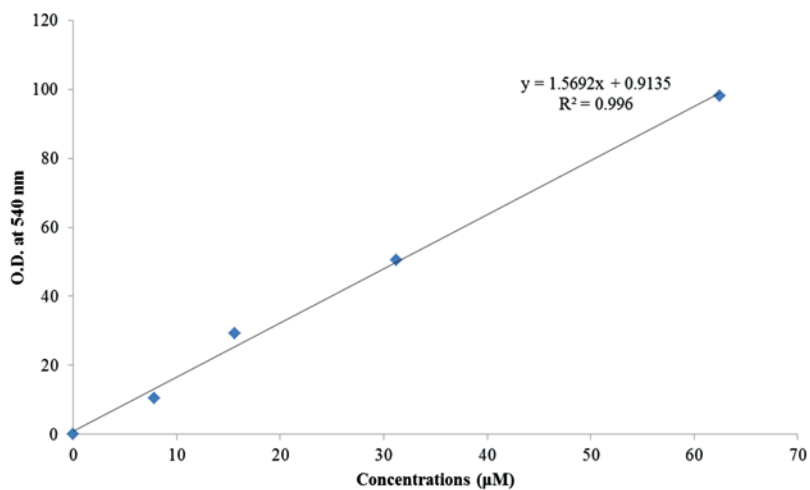

Fig. 2 Calibration curve for DPPH radical scavenging activity probe as a function of standard trolox concentrations

Table 3 Antioxidant activity of taurine by FRAP assay

Concentrations (μg/mL)	μM TE
125	6.34 ± 0.25
250	8.61 ± 0.35
500	9.49 ± 0.88
1,000	17.51 ± 0.55

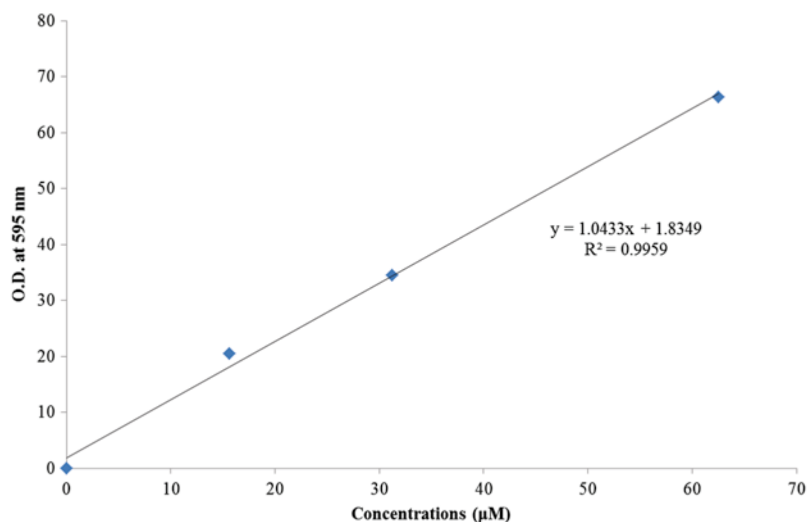

Fig. 3 Calibration curve for FRAP assay probe as a function of standard trolox concentrations

Table 4 Antioxidant activity of taurine by FRAP assay

Concentrations (μg/mL)	μM TE
125	6.18 ± 0.25
250	29.16 ± 0.30
500	45.13 ± 0.22
1,000	52.09 ± 1.00

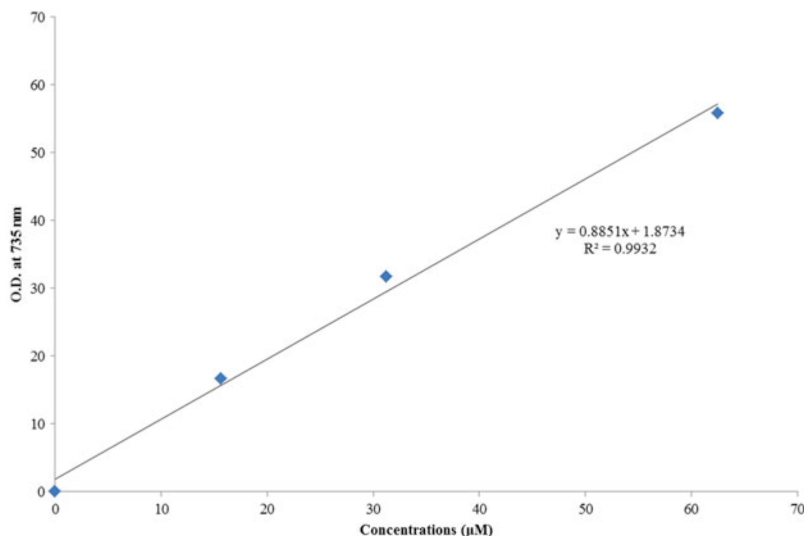

Fig. 4 Calibration curve for ABTS assay probe as a function of standard trolox concentrations

3.5 *Anti-breast Cancer Activity of Taurine*

The anticancer activity of taurine was evaluated analyzing cell viability rates using MTT assay. As shown in Fig. 5, taurine significantly stimulated breast cancer cells death in a dose-dependent manner at concentrations of 0.125–1.0 mM.

4 Discussion

Numerous in vitro studies have been conducted to evaluate the total antioxidant capacity (TAC) of food products. So far, however, there is no official standardized method, and therefore it is recommended that each evaluation should be made with various oxidation conditions and different methods of measurement (Frankel and Meyer 2000). The methods for measuring antioxidant capacity are basically classified into two groups, depending on the reaction mechanism: methods based on hydrogen atom transfer (HAT) and methods based on electron transfer (ET) (Huang

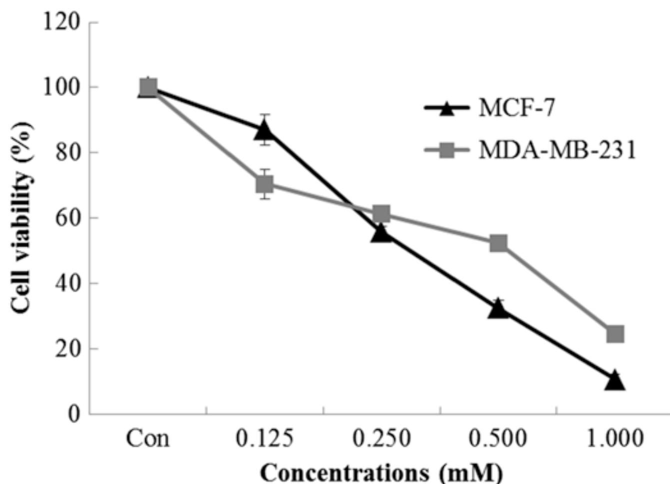

Fig. 5 Cell viability analysis of taurine on two types of human breast cancer cells; MCF-7 and MDA-MB-231. Cells were incubated with various concentrations of taurine for 24 h followed by MTT for 4 h, and then 100 μL of isopropanol was added to dissolve the formazan crystals

et al. 2005). The majority of HAT-based assays apply a competitive scheme, in which antioxidant and substrate compete for thermally generated peroxyl radicals through the decomposition of azo-compounds. ET based assays measure the capacity of an antioxidant in the reduction of an oxidant, which changes color when reduced. The degree of color change is correlated with the sample's antioxidant concentrations. Trolox equivalent antioxidant capacity (TEAC) or ABTS and ORAC assays are the most popularly used ET and HAT methods, respectively. The ABTS assay is based on scavenging of the ABTS•+ radical cation by the antioxidants present in a sample. The ABTS•+ radical typically has a bluish-green color with maximum absorbance values at 645, 735 and 815 nm (Re et al. 1999). When there are antioxidant compounds in the reaction medium, they capture the free radical, which is translated into a loss of color and therefore a reduction in absorbance, corresponding quantitatively to the concentration of antioxidants present. The ORAC method, developed initially by Cao et al. (1993) consists of measuring the decrease in the fluorescence of a protein as a result of the loss of its conformation when it suffers oxidative damage caused by a source of peroxyl radicals (ROO·). The method measures the ability of the antioxidants in the sample to protect the protein from oxidative damage. In this study beside ABTS and ORAC, we also used other ET based-methods; DPPH radical and FRAP for the antioxidant activity. Four types of methods present the antioxidant activities with the values of 29.97 μM, 15.15 μM, 17.51 μM, and 52.09 μM TE at 1,000 μg/mL by ORAC, DPPH radical scavenging activity, FRAP, and ABTS, respectively (Tables 1, 2, 3, and 4). Especially important, since this is the first time evaluation of ORAC from taurine was performed and it gives basic data for further study. In ORAC for taurine, the activity was similar with zeaxanthin, and higher than ascorbic acid, gallic acid, and albumin

(Zulueta et al. 2009). Except ORAC, most of results for DPPH, FRAP, and ABTS were presented as IC_{50} or % of scavenging activity, therefore, there were difficulties to compare with other reports.

The antioxidant properties have generated great interest in researchers. The properties are commonly postulated to play an important role in preventing diseases caused by oxidative stress, such as cancer and aging. For this reason, we also estimated the anti-breast cancer activity of taurine using MTT assay. MCF-7 cells were more sensitive than MDA-MB-231 cells to taurine treatment on cell viability (Fig. 5). We assume the reason of the sensitivity was due to hormone dependency, however further investigation is needed to determine/elucidate the precise mechanism.

In summary, we evaluated the antioxidant activities using ORAC, DPPH, FRAP, and ABTS assays and anti-breast cancer activity on two types of human breast cancer cells; MCF-7 and MDA-MB-23. These results show that taurine possesses potent antioxidant activity and potential as an anti-breast cancer agent.

5 Conclusion

From these multiple chemical and biological in vitro investigations, it was proved that taurine scavenged reactive oxygen species free radicals and induced various cancer cells death. This proof indicates the possibility for taurine to be used as a new source of natural antioxidants and preventive agent for breast cancer.

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Effect of Taurine on *In Vitro* Migration of MCF-7 and MDA-MB-231 Human Breast Carcinoma Cells

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Abbreviations

MMPs	Matrix metalloproteinases
TIMPs	Tissue inhibitors of metalloproteinases
VEGF	Vascular endothelial growth factor

1 Introduction

Breast cancer mortality was reduced by 25 % over the last two decades due to developed treatment strategies (Yue et al. 2013). However, its incidence in the United States remains the highest of all cancers in women (Siegel et al. 2012). In addition, there has been a recent trend in Asia towards increasing incidence of breast cancer, reporting a more rapid increase than high-risk countries (Jemal et al. 2010a, b).

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Decline in incidence and eventual extermination of breast cancer will need improvement or a more effective plan for prevention.

Estradiol (E2) administration causes breast cancer in various animal models and anti-estrogens abrogate this effect (Zumoff 1998; Hollingsworth et al. 1998). Long term exposure to estrogens is associated with an increased risk of breast cancer (Yue et al. 2013). Estrogens could cause de novo breast cancer though either hormone-dependent or independent mechanisms. Through actions mediated by its receptor, estradiol enhances cell proliferation, a factor causally related to breast cancer development. Preston-Martin et al. described in detail the mechanisms whereby enhanced cell proliferation facilitates cancer development (Preston-Martin et al. 1990). Clinical and experimental data suggest the possibility that hormone-independent effects of E2 may also be mechanistically involved in de novo breast cancer development (Rebbeck et al. 2009). Tumors are frequently found to be a heterogeneous mixture of estrogen receptor-negative and estrogen receptor-positive cell types (Robinson et al. 1990; King et al. 1985; Shimada et al. 1985). Anti-estrogens are effective in controlling the growth of estrogen-dependent tumors. However, these agents cannot control hormone-independent tumors (Robinson and Jordan 1989). Accordingly, the accurate pathways responsible for estrogen related carcinogenesis are not well established. The most widely accepted theory holds that estradiol (E2), acting through estrogen receptor alpha (ERα), stimulates cell proliferation and initiates mutations arising from replicative errors occurring during pre-mitotic DNA synthesis. The promotional effects of E2 then support the growth of cells harboring mutations. Therefore, we used two types of breast cancer cells; MCF-7 for estrogen receptor-dependent and MDA-MB-231 for independent cells in this study.

Taurine (β-ethanesulfonic acid), the sulfur-containing free amino acid, is the most plentiful amino acid in mammalian tissue, reaching concentrations as much as 5–20 μmol/g wet wt (Chapman et al. 1993; Chesney 1985). To the best of our knowledge, there is little investigation of the effect of taurine on metastasis in hormone-dependent and independent breast cancer cells. In this study, therefore, we elucidated the effect of taurine on anti-metastasis potentials in two types of breast cancer cells; hormone-dependent and independent.

2 Methods

2.1 Materials

MCF-7, hormone-dependent, and MDA-MB-231, hormone-independent human breast cancer cells were obtained from Korean Cell Line Bank, respectively (Seoul, Korea). Taurine and E2 was purchased from Sigma (St. Louis, MO, USA). Antibodies for primary antibodies and the peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

2.2 Cell Culture

The human breast cancer cell line, MCF-7 and MDA-MB-231 were cultured in DMEM medium, supplemented with 10 % FBS, 1 % penicillin/streptomycin in a 5 % CO_2 atm at 37 °C. The cells were seeded at a density of 3.5×10^5 cells well in a 13 cm well culture dish. After 24 h, the cells were treated with 0.125, 0.250, and 0.500 mM of taurine in medium. Cells were treated with taurine for 24 h and then harvested.

2.3 Gap Closure Cell Migration Assay

Radius™ 24-well cell migration assay originated from Cell Biolabs, Inc (San Diego, USA). To determine which wells would be assayed, 500 μl of Radius™ gel pretreatment solution was slowly added to each well by careful pipetting down the wall of the well. The plate was then covered and incubated at room temperature for 20 min. Radius™ gel pretreatment solution was carefully aspirated the from the wells and 500 μl of Radius™ wash solution was added to each well. The cells were harvested and resuspended in culture medium at 0.2×10^6 cell/ml. Radius™ wash solution was carefully aspirated the from the wells and 500 μl of the cell suspension was added to each well by careful pipetting down the wall of the well. The plate was transferred to a cell culture incubator for 24 h to allow firm attachment. After 24 h, the media were aspirated from each well and washed three times with 0.5 ml of fresh medium. Sufficient 1× Radius™ gel removal solution was prepared for all wells by diluting the stock 1:100 in culture medium. The media were aspirated from the wells and 0.5 ml of 1× Radius™ gel removal solution was added to each well and washed three times with 0.5 ml of fresh medium. After the final washing was complete, 1 ml of complete medium and taurine were added to each well and a photo was taken on 0 h, 8 h, 24 h and 48 h, respectively. To compare the differences in migratory gap, images were captured at the same size, and gap closure was determined after the indicated times (0, 8, 24 and 30 h) using CellProfiler™ software (Broad Institute, MA, USA).

2.4 Western Blot

After the indicated treatments, cells were harvested in PBS and lysed in RIPA buffer (150 mM NaCl, 0.5 % deoxycholate, 0.1 % nonidet P-40, 0.1 % SDS and 50 mM Tris) containing protease inhibitors (50 mg/ml phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, 5 mg/ml leupeptin, 0.1 mg/ml NaF, 1 mM DTT, 0.1 mM sodium orthovanadate and 0.1 mM b-glycerophosphate). Total cellular proteins were quantified by the Bradford procedure and equal amounts of proteins were mixed with loading buffer (25 % glycerol, 0.075 % SDS, 1.25 ml of 14.4 M 2-mercaptoethanol, 10 % bromophenol blue and 3.13 % stacking gel buffer) and fractionated by gel electrophoresis on

gradient gels (Novex, CA, USA). Rainbow marker (Novex, CA, USA) was used as the molecular weight standard. Proteins were transferred to nitrocellulose membranes (Novex, CA, USA) and blocked for 1.5 h with clear milk (Thermo Scientific, IL, USA). Blots were subsequently incubated with primary antibodies in 1× TBST for 1.5 h. Goat anti-rabbit or goat anti-mouse horseradish peroxidase conjugated secondary antibodies (Santa Cruz Biotechnology, TX, USA) were used at 1:5,000 dilution in 1× TBST. Blots were treated with Western Lightning Western Blot Chemiluminescence Reagent (Advansta, CA, USA) and the proteins were detected by autoradiography (Fujifilm, Japan). Equal protein loading was ascertained by β-actin bodies.

2.5 Statistical Analysis

All data are presented using the mean ± SE and the data sourced from at least three experiments. Statistical analyses were performed using SAS statistical software (SAS Institute, Cary, NC, USA). Treatment effects were analyzed using one-way analysis of variance, followed by Dunnett's multiple range tests. The result of $p < 0.05$ was used to indicate significance.

3 Results

3.1 The Effect of Taurine on Hormone-Dependent Human Breast Cancer Cells Migration

To examine the effect of taurine on hormone-dependent breast cancer cells, MCF-7 migration, we performed gap closure assay using a Radius™ 24 well. To compare the differences in migratory gap, images were captured at the same size, and gap closure was determined after the indicated times (0, 8, 24 and 30 h) compared to control and E2 group. After 30 h, the gap was closed approximately 35 % in E2-treated cells. As shown in Fig. 1, taurine significantly reduced the cell motility, compared with E2 alone-treated cells.

3.2 The Effect of Taurine on Hormone-Independent Human Breast Cancer Cells Migration

To examine the effect of taurine on hormone-independent breast cancer cells, MDA-MB-231 migration, we performed gap closure assay using a Radius™ 24 well. To compare the differences in migratory gap, images were captured at the same size, and gap closure was determined after the indicated times (0, 8, 24 and 30 h) compared to control and E2 group. After 30 h, the gap was closed in approximately 80 % in E2-treated cells. As shown in Fig. 2, taurine significantly reduced the cell motility, compared with E2 alone-treated cells.

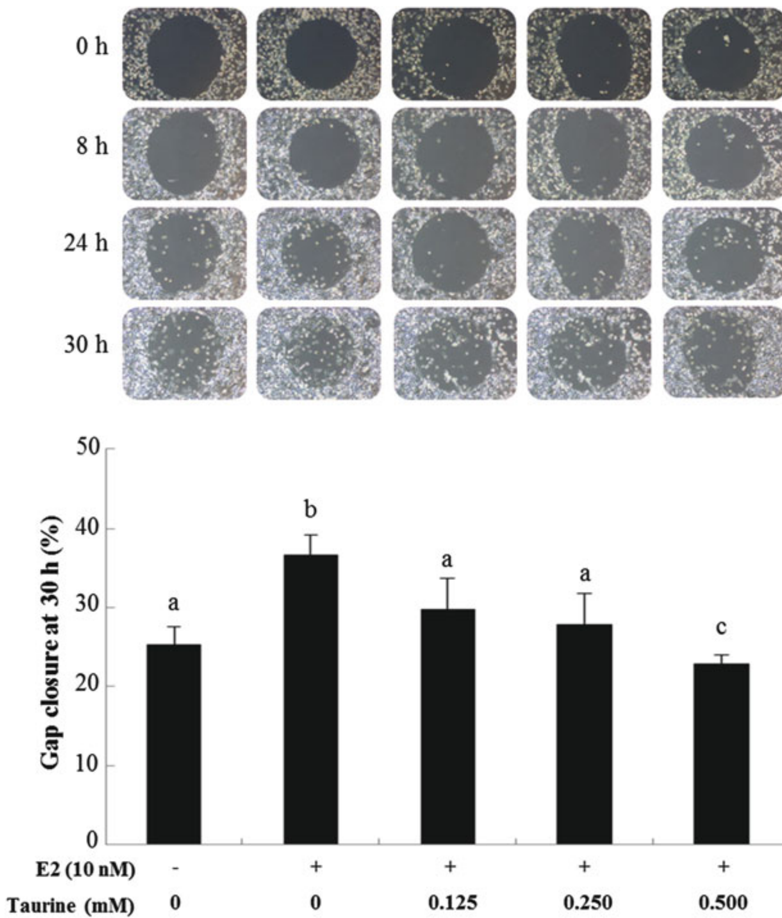

Fig. 1 The effect of taurine on the gap closure ability in hormone-dependent breast cancer cells, MCF-7. Allowed to grow for indicated time in the presence or absence of E2 and different concentrations of taurine. The gaps covered by the cells were measured by CellProfiler™. The gap represents the mean of three individual experiments performed in triplicate. Values not sharing a common letter are significantly different at $P < 0.05$ by Dunnett's multiple range tests

3.3 The Effect of Taurine on the Expression of Migration-Related Genes in Hormone-Dependent Human Breast Cancer Cells

The expression of migration-related genes such as, VEGF, MMP-9, TIMP-1, and TIMP-2 were investigated by Western blot after treatment with E2 for 24 h. Treatment of MCF-7 cells with taurine significantly decreased VEGF expression (Fig. 3a, b). However, taurine did not affect the protein expression of MMP-9 (Fig. 3a, c). Meanwhile, TIMP-1 and TIMP-2 significantly and dose-dependently increased the protein expressions in taurine-treated cells (Fig. 3a, d, e).

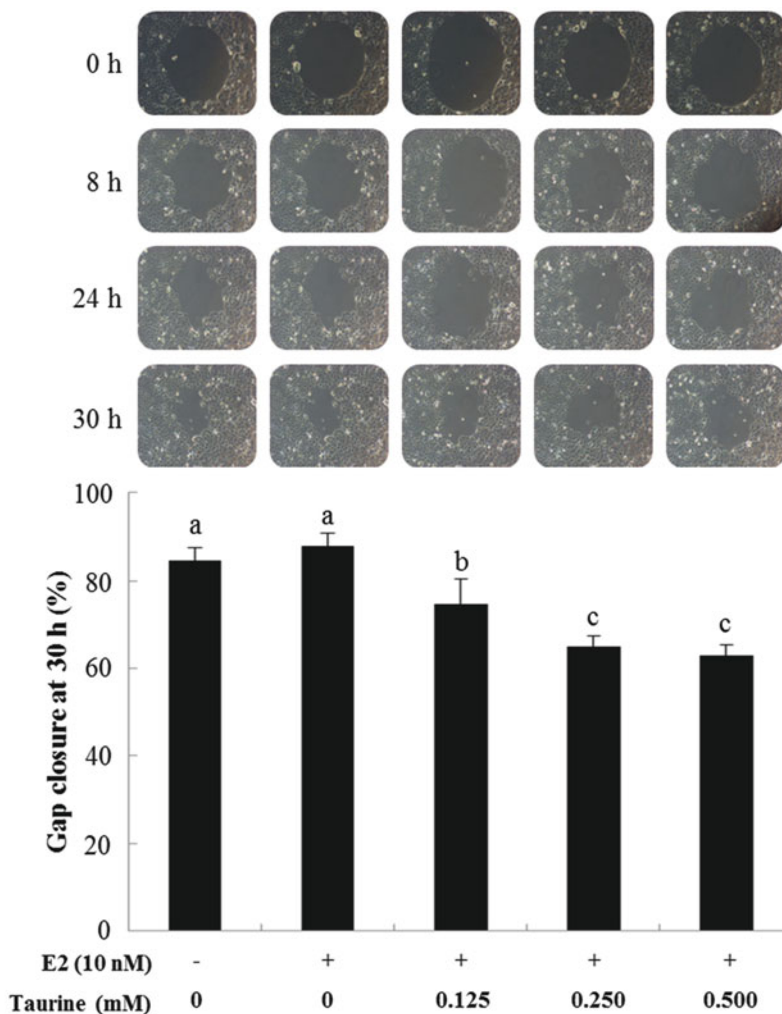

Fig. 2 The effect of taurine on the gap closure ability in hormone-dependent breast cancer cells, MDA-MB-231. Allowed to grow for indicated time in the presence or absence of E2 and different concentrations of taurine. The gaps covered by the cells were measured by CellProfiler™. The gap represents the mean of three individual experiments performed in triplicate. Values not sharing a common letter are significantly different at $P<0.05$ by Dunnett's multiple range tests

3.4 The Effect of Taurine on the Expression of Migration-Related Genes in Hormone-Independent Human Breast Cancer Cells

The expression of migration-related genes such as, VEGF, MMP-9, TIMP-1, and TIMP-2 were investigated by Western blot after treatment with E2 for 24 h. Treatment of MDA-MB-231 cells with taurine significantly decreased VEGF

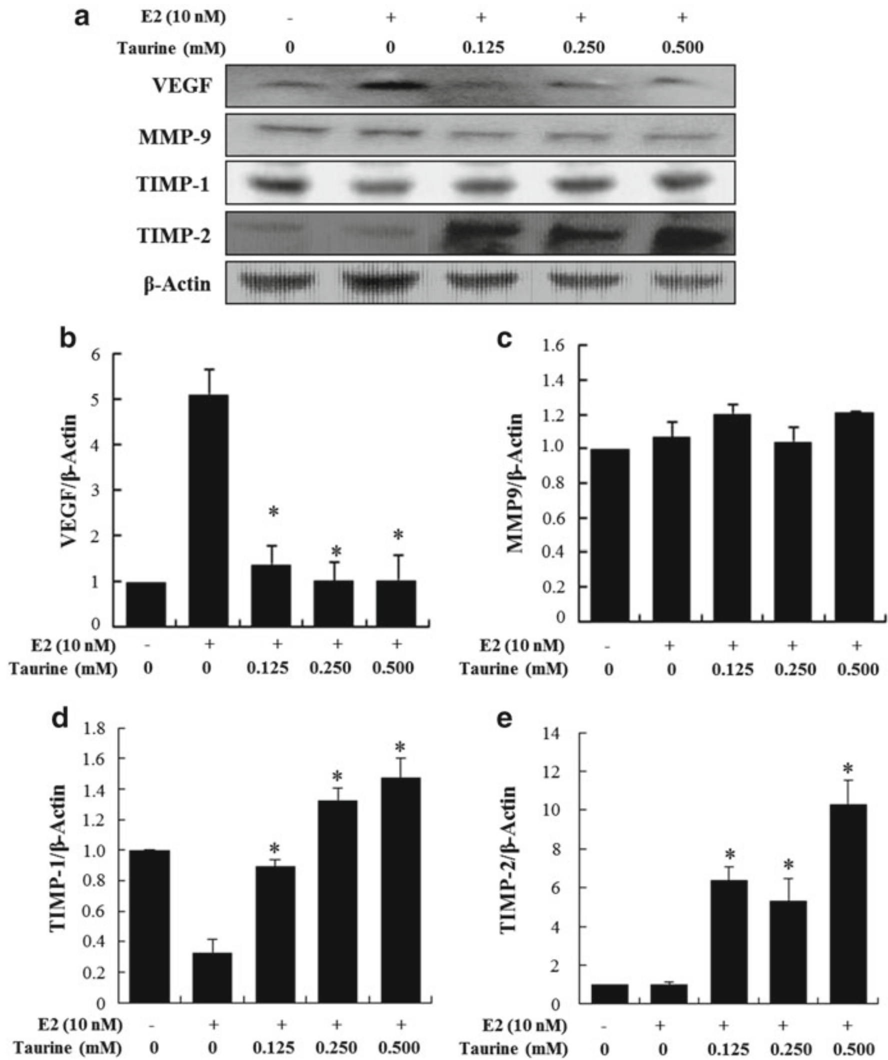

Fig. 3 (**a**) Protein expression of migration-related genes in blots. Expression of VEGF, (**b**) MMP-9, (**c**) TIMP-1, (**d**) and TIMP-2 (**e**) were nomalized by β-actin, and quantified by Image Guage (Fujifilm, Japan)

expression at 0.125 and 0.250 mM (Fig. 4a, b). However, 0.500 mM of taurine did not affect the protein expression of VEGF (Fig. 4a, b). The expression of MMP-9 was decreased by taurine. Meanwhile, taurine attenuated the expression of TIMP-1 at 0.25 mM only (Fig. 4a, d). The expression of TIMP-2 was not significantly changed (Fig. 4a, e).

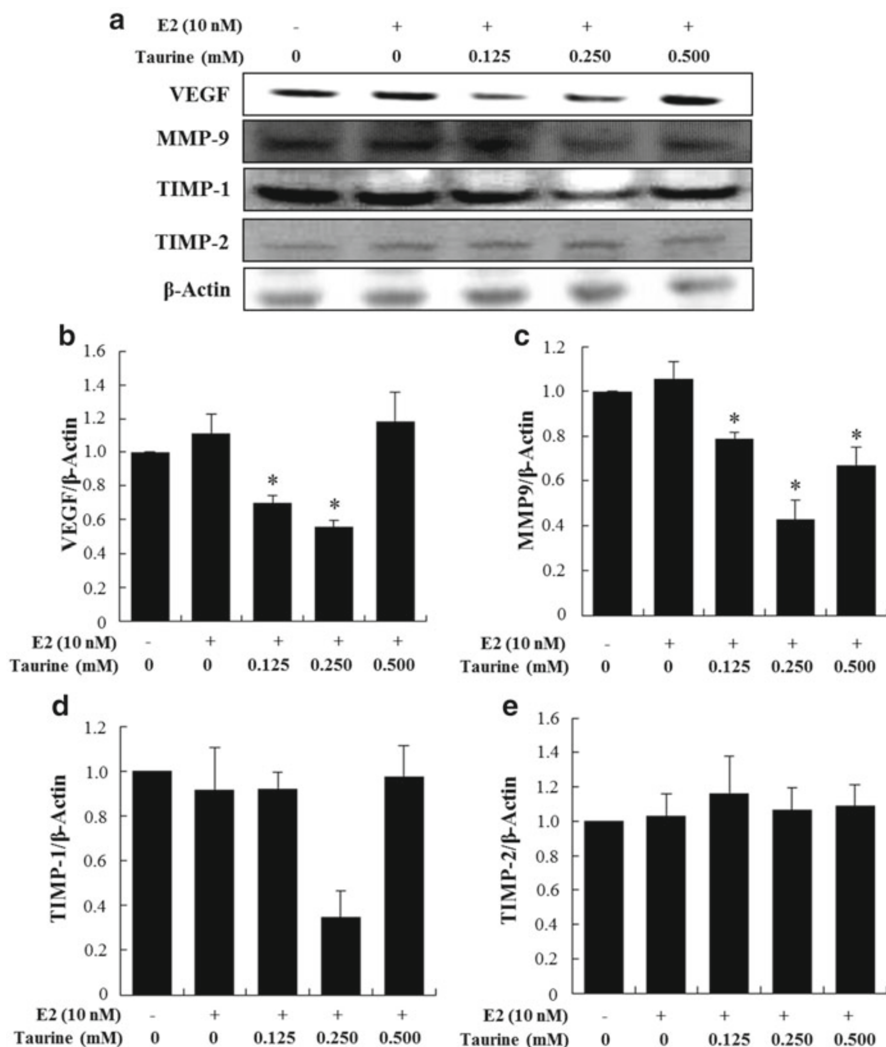

Fig. 4 (**a**) Protein expression of migration-related genes in blots. Expression of VEGF, (**b**) MMP-9, (**c**) TIMP-1, (**d**) and TIMP-2 (**e**) were nomalized by β-actin, and quantified by Image Guage (Fujifilm, Japan)

4 Discussion

Breast cancer is related to reproductive factors (Kobayashi et al. 2012), genetic background (Cao et al. 2013; Xia et al. 2014), body composition (Irwin et al. 2009; Schmitz et al. 2005), nutrition (Hauner et al. 2011; Yaw et al. 2014) and obesity (Demark-Wahnefried et al. 2012; Sangrajrang et al. 2013). Currently,

three major strategies have been linked to an increase in breast cancer risk: obesity, lack of physical exercise, and high levels of saturated dietary fat (Alegre et al. 2013). Although progress has been made in reducing incidence and mortality rates and improving survival, cancer still accounts for more deaths than heart disease (Jemal et al. 2010a, b). In addition, breast cancer is one of the leading causes of death in women because of its high metastasis and invasion of the lymph nodes, lungs, and bones, even the brain, during the terminal phase of cancer (Marmot et al. 2013; Niikura et al. 2013). A series of treatments have been utilized to relieve cancer-related symptoms, delay cancer progression and prolong and improve the quality of life; however, metastatic breast cancer still remains an incurable condition (Harding et al. 2013; Eckhardt et al. 2012). Therefore, in this study, we investigate the effect of taurine on migration and migration-related gene expression in breast cancer cells.

MCF-7 cells were more sensitive than MDA-MB-231 cells to E2 treatment on migration rate (Fig. 1 and 2). In addition, on the migration rates at 30 h, MDA-MD-231 cells were much higher than MCF-7 cells. These results correlated with previous reports (Robinson et al. 1990). Meanwhile, angiogenesis is an essential process of cancer cell growth and migration, therefore, we investigated the expression of angiogenesis related genes. VEGF is considered to be the main factor to promote angiogenesis (Cho et al. 2001). In addition, MMPs can degrade almost all extracellular matrix (ECM) components and then provide a channel for cancer cells migration. MMP-9 is one of the key proteinase of MMPs in the degradation of ECM (Zhou et al. 2014). We also estimated the expression of TIMPs which inhibit MMPs activity. Firstly, protein expressions of VEGF, MMP-9, TIMP-1 and TIMP-2 for 24 h in hormone-dependent breast cancer cells, MCF-7, were estimated. As shown in Fig. 3, treatment of MCF-7 cells with taurine significantly decreased the expressions of VEGF compared to the E2 alone-treated group. Contrarily, the expression of TIMP-1 and TIMP-2 was increased in the DHT-treated with velvet antler group. We also performed the same experiment on hormone-independent cells, MDA-MB-231 (Fig. 4). VEGF and MMP-9 were decreased by taurine against E2 alone-treated group. Meanwhile, taurine attenuated the expression of TIMP-1 at 0.25 mM.

5 Conclusion

In the current work, we performed several experiments to prove the effect of taurine on breast cancer cells migration and the related-genes expressions. Taurine reduced the migration rates and the expression of the related-genes. On the contrary, taurine induced TIMP-1. These results suggest that further investigation is needed to elucidate the mechanism of the anti-migration effect of taurine.

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Effect of Taurine on Prostate-Specific Antigen Level and Migration in Human Prostate Cancer Cells

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Abbreviations

MMPs	Matrix metalloproteinases
PSA	Prostate-specific antigen
TIMPs	Tissue inhibitors of metalloproteinases
VEGF	Vascular endothelial growth factor

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1 Introduction

Taurine, 2-aminoethanesulfonic acid was first isolated more than 150 years ago from ox bile in 1827 by German scientists Friedrich Tiedemann and Leopold Gmelin (Tiedemann and Gmelin 1827). Taurine has been implicated in neurodegenerative disease, antioxidant property, atherosclerosis and coronary heart disease (Olive 2002; Green et al. 1991; Zhang et al. 2004; Choi et al. 2006). Nevertheless, there aren't many reports on anti-cancer property of taurine (Kirk and Kirk 1993; Yanagita et al. 2008; Chatzakos et al. 2012).

Prostate cancer is the most frequent malignancy in men reaching $8 billion in expenses with an average cost of $81,658 per patient, from diagnosis till death in the US alone (Racioppi et al. 2012; Klein and Thompson 2012) with ~350,000 new cases diagnosed annually in Europe (Siegel et al. 2012; Jemal et al. 2009). Prostate cancer is also the most common cancer in developed world with increasing rates in the developing world (Baade et al. 2009). Over the last 25 years, the number of men diagnosed with prostate cancer each year has increased by 30 % (Wingo et al. 2003). Therefore, a number of agents are currently being investigated for the prevention of prostate cancer (Klein and Thompson 2012).

Prostate specific antigen (PSA), an enzyme of 30 kDa grouped in the kallikrein family and also known as kallikrein-related peptidase 3 (KLK3) is synthesized to high levels by normal and malignant prostate epithelial cells. Therefore, it is the key biomarker currently applied for early diagnosis of prostate cancer (Luigi et al. 2014). The ability of PSA to process a number of growth regulatory proteins that are important in cancer growth and survival (such as insulin-like growth factor binding protein, parathyroid hormone-related protein, latent transforming growth factor-beta 2 as well as extracellular matrix components, like fibronectin and laminin) (Cohen et al. 1992; Iwamura et al. 1996), indeed PSA can facilitate tumor growth and metastasis dissemination (Williams et al. 2007; Webber et al. 1995).

The tumor metastasis is associated with a multigene and multistep process with the participation of various metastasis-related genes. Degradation of the j matrix (ECM) by MMPs is an essential mechanism in tumor metastasis. Studies have revealed that MMPs are the common and crucial target effectors for many oncogenes and tumor suppressor genes facilitating tumor metastasis (Shuman Moss et al. 2012). MMP9, a key member of the MMPs, plays a vital role in cancer metastasis process. In addition, angiogenesis is also required for formation of tumor metastasis. Tumors that have become neovascularized often express increased levels of proangiogenic proteins, such as VEGF. However, to the best of our knowledge, there is no scientific report for PSA, MMP9 and VEGF. In the present study, therefore, we investigated the effects of taurine on PSA, MMPs and VEGF expression in vitro using human prostate cancer cells, LNCaP and PC-3.

2 Methods

2.1 Materials

LNCaP, androgen-dependent human prostate cancer cells were obtained from Korean Cell Line Bank (Seoul, Korea; KCLB numbers: 21740). Taurine and DHT (dihydrotestosterone) were purchased from Sigma (St. Louis, MO, USA). Antibodies for primary antibodies and the peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

2.2 Cell Culture

The human prostate cancer cell line, LNCaP and PC-3 were cultured in RPMI 1640 medium, supplemented with 10 % FBS, 1 % penicillin/streptomycin in a 5 % CO_2 atm at 37 °C. The cells were seeded at a density of 3.5×10^5 cells well in a 13 cm well culture dish. After 24 h, the cells were treated with 0.125, 0.250, 0.500, and 1.000 mM of taurine in medium. Cells were treated with taurine for 24 h and then harvested.

2.3 Cell Viability

Cell viability was determined by 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay in 96-well plates as previously described. Cells were incubated with various concentrations of taurine for 48 h followed by MTT for 4 h, and then 100 μL of isopropanol (in 0.04 N-hydrochloric acid) was added to dissolve the formazan crystals. The absorbance was read at 570 nm using the Anthos 2010 spectrophotometer (Salzburg, Austria). Cell viability was calculated as being the relative absorbance compared to control (Kim et al. 2002).

2.4 Gap Closure Cell Migration Assay

Radius™ 24-well cell migration assay originated from Cell Biolabs, Inc (San Diego, USA). To determine which wells would be assayed, 500 μl of Radius™ gel pretreatment solution was slowly added to each well by careful pipetting down the wall of the well. The plate was covered and incubated at room temperature for 20 min. Radius™ gel pretreatment solution was carefully aspirated from the wells, 500 μl of Radius™ wash solution was added to each well. The cells were harvested and resuspended in culture medium at 0.2×10^6 cell/ml. Radius™ wash solution was carefully

aspirated from the wells, 500 μl of the cell suspension was added to each well by careful pipetting down the wall of the well. The plate was transferred the to a cell culture incubator for 24 h to allow firm attachment. After 24 h, the media from each well were aspirated and washed three times with 0.5 ml of fresh medium. Sufficient 1× Radius™ gel removal solution was prepared for all wells by diluting the stock 1:100 in culture medium. The media were aspirated from the wells and 0.5 ml of 1× Radius™ gel removal solution was added to each well and washed three times with 0.5 ml of fresh medium. After the final washing was complete, 1 ml of complete medium and taurine were added to each well, and a photo was taken on 0 h, 8 h, 24 h and 48 h, respectively. To compare the differences in migratory gap, images were captured at the same size, and gap closure was determined after the indicated times (0, 8, 24 and 48 h) using CellProfiler™ software (Broad Institute, MA, USA).

2.5 Western Blot

After the indicated treatments, cells were harvested in PBS and lysed in RIPA buffer (150 mM NaCl, 0.5 % deoxycholate, 0.1 % nonidet P-40, 0.1 % SDS and 50 mM Tris) containing protease inhibitors (50 mg/ml phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, 5 mg/ml leupeptin, 0.1 mg/ml NaF, 1 mM DTT, 0.1 mM sodium orthovanadate and 0.1 mM b-glycerophosphate). Total cellular proteins were quantified by the Bradford procedure and equal amounts of proteins were mixed with loading buffer (25 % glycerol, 0.075 % SDS, 1.25 ml of 14.4 M 2-mercaptoethanol, 10 % bromophenol blue and 3.13 % stacking gel buffer) and fractionated by gel electrophoresis on gradient gels (Novex, CA, USA). Rainbow marker (Novex, CA, USA) was used as the molecular weight standard. Proteins were transferred to nitrocellulose membranes (Novex, CA, USA) and blocked for 1.5 h with clear milk (Thermo Scientific, IL, USA). Blots were subsequently incubated with primary antibodies in 1× TBST for 1.5 h. Goat anti-rabbit or goat anti-mouse horseradish peroxidase conjugated secondary antibodies (Santa Cruz Biotechnology, TX, USA) were used at 1:5,000 dilution in 1× TBST. Blots were treated with Western Lightning Western Blot Chemiluminescence Reagent (Advansta, CA, USA) and the proteins were detected by autoradiography (Fujifilm, Japan). Equal protein loading was ascertained by β-actin bodies.

2.6 Statistical Analysis

All data are presented using the mean ± SE and the data sourced from at least three experiments. Statistical analyses were performed using SAS statistical software (SAS Institute, Cary, NC, USA). Treatment effects were analyzed using one-way analysis of variance, followed by Dunnett's multiple range tests. For the results $p < 0.05$ was used to indicate significance.

3 Results

3.1 The Effect of Taurine on Viability of Human Prostate Cancer Cells

The antiproliferative activity of taurine was evaluated using MTT assay. As shown in Fig. 1, taurine significantly stimulated prostate cancer cells death in a dose-dependent manner at concentrations of 0.125–1.0 mM. We omitted 1.0 mM from the results, which showed 30 % below cell viability from the next experiments.

3.2 The Effect of Taurine on Human Prostate Cancer Cells Migration

To examine the effect of taurine on cell migration, we performed gap closure assay using a Radius™ 24 well. To compare the differences in migratory gap, images were captured at the same size, and gap closure was determined after the indicated times (0, 8, 24 and 48 h) compared to control and DHT group. In LNCaP cells, after 48 h, the gap was closed in approximately 50 % in DHT-treated cells (Fig. 2a). In addition, in PC-3 cells, after 8 h, the gap was closed in approximately 60 % in DHT-treated cells (Fig. 2b). As shown in Fig. 2, taurine significantly reduced cell motility, compared with DHT alone-treated cells in both LNCaP and PC-3 cells.

3.3 The Effect of Taurine on the Expression of PSA and Migration-Related Genes

First, protein expression of PSA was investigated by Western blot after treatment with DHT (1 nM) for 48 h. Treatment of LNCaP cells with taurine significantly decreased PSA expression (Fig. 3a, b). Treatment of PC-3 cells with taurine significantly decreased PSA expression (Fig. 3f, g). In addition, migration related genes were also estimated. As shown in Fig. 3a–c, taurine significantly suppressed protein expression of MMP-9 in a dose-dependent fashion in LNCaP cells. Moreover, TIMP-1 and TIMP-2 which are naturally occurring inhibitors of MMP-9, significantly and dose-dependently increased the protein expressions in taurine-treated cells in LNCaP (Fig. 3a, d, e). These data suggest that taurine suppresses the migratory condition by regulating the levels of TIMP-1 and TIMP-2 in androgen-dependent human prostate cancer cells, LNCaP. In addition, Treatment of PC-3 cells with taurine significantly decreased PSA expression (Fig. 3f, g). Moreover, VEGF which is considered to be the main factor promoting angiogenesis was significantly and dose-dependently attenuating the protein expressions in taurine-treated cells (Fig. 3f, h). These data suggest that taurine suppresses the migratory condition by regulating the levels of VEGF in androgen-resistant human prostate cancer cells, PC-3.

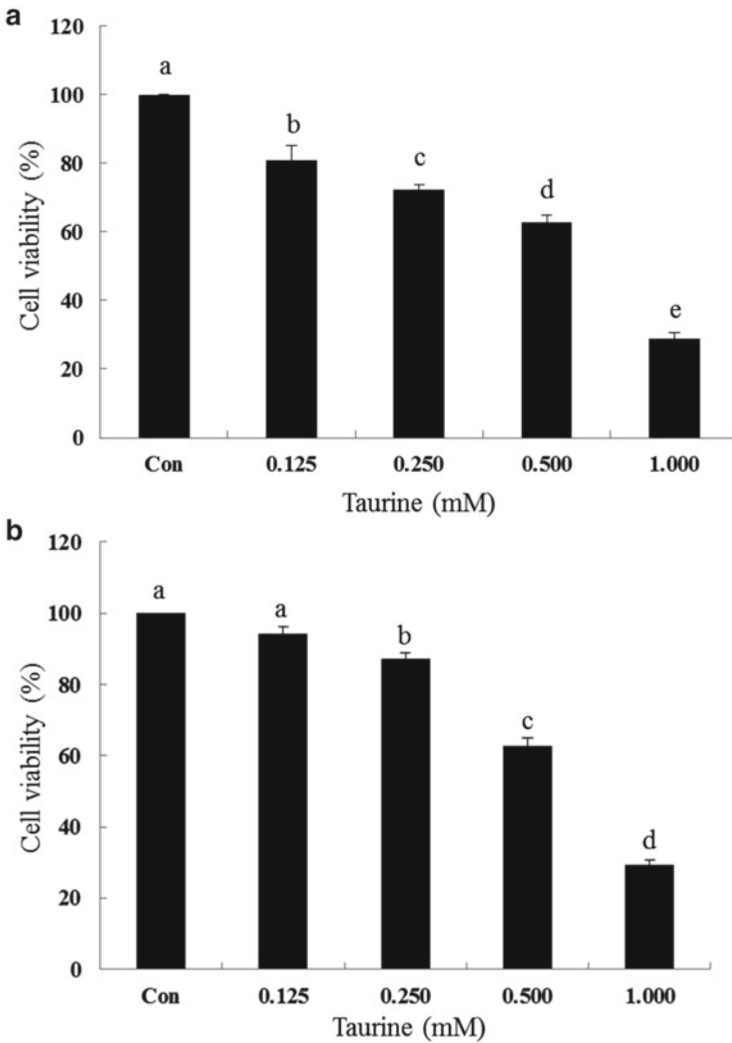

Fig. 1 Cell viability analysis of taurine on human prostate cancer cells, LNCaP (**a**) and PC-3 (**b**). Prostate cancer cells were incubated with various concentrations of taurine for 48 h followed by MTT for 4 h, and then 100 μl of isopropanol was added to dissolve the formazan crystals. Each value represents the mean ± SEM. Values not sharing a common letter are significantly different at $P < 0.05$ by Dunnett's multiple range tests

4 Discussion

The sulfur-containing -amino acid, taurine, is the most plentiful free amino acid in cardiac and skeletal muscle. Recently, several studies have investigated that taurine exhibits anti-proliferative and antineoplastic effects in prostate cancer cells

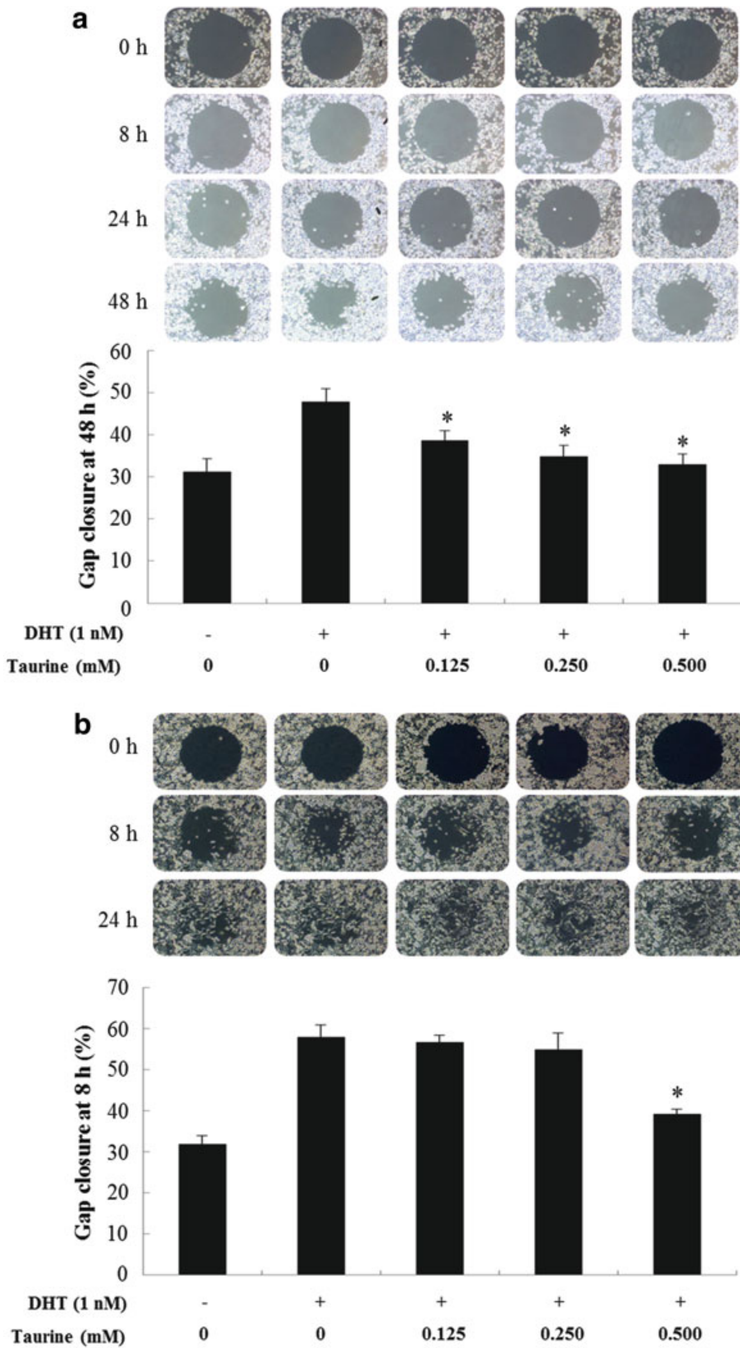

Fig. 2 The effect of taurine on the gap closure ability in human prostate cancer cells, LNCaP (**a**) and LNCaP (**b**). Allowed to grow for indicated time in the presence or absence of DHT and different concentrations of taurine. The gap covered by the cells was measured by CellProfiler™. The gap represents the mean of three individual experiments performed in triplicate. *$P<0.05$, statistical significance compared with DHT alone-treated cells

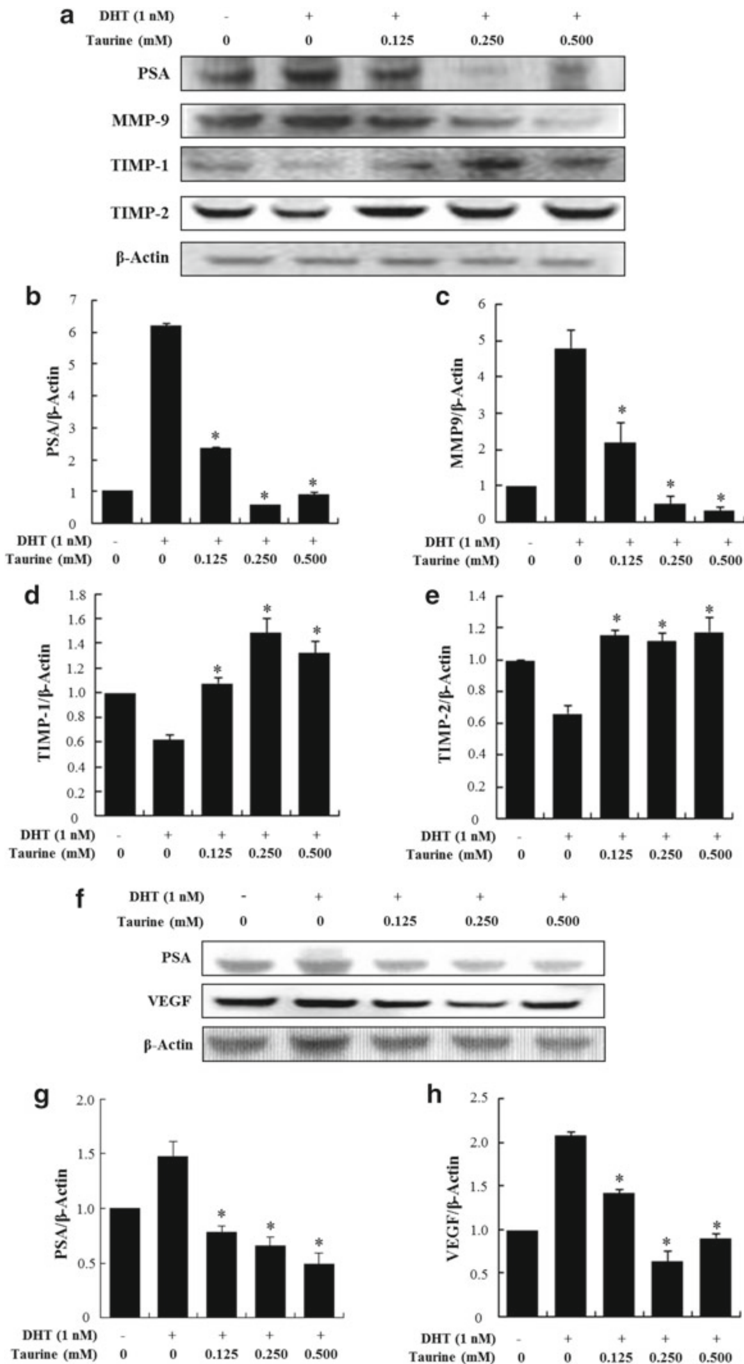

Fig. 3 The effect of taurine on PSA and migration-related genes in human prostate cancer cells, LNCaP (**a**–**e**) and PC-3 (**f**–**h**). Cells were treated with or without DHA and various concentrations of taurine for 48 h. The protein levels from whole-cell lysates were analyzed by Western blot. β-actin was used as a loading control. The blot represents the mean of three individual experiments performed in triplicate. *$P < 0.05$, statistical significance compared with DHT alone-treated cells

(Chatzakos et al. 2012; Darnowski et al. 2004; Zhang et al. 2008). The regulation of cell growth is a homeostatic balance between stimulatory and inhibitory signals. The negative growth control by tumor suppressor genes, differentiation factors, and programmed cell death (apoptosis) is commonly targeted mechanism exploited for strategies in the treatment of malignancies and other diseases. Among them, apoptosis is a highly attractive and widely studied area to search for more effective agents for treatment of human cancers. A wide variety of *in vivo* and *in vitro* studies published in recent years suggested that many chemotherapeutic agents could induce apoptotic cell death in different cancer cells (McCloskey et al. 1996). For this reason, the anticancer activity of taurine was evaluated for apoptosis using MTT assay. As shown in Fig. 1, taurine stimulated apoptosis in a dose-dependent manner. From the results, we decided upon the concentrations of taurine for the next experiments.

Cellular adhesion and migration are important features of cancer progression and therefore a potential target for cancer interception (Elgass et al. 2014). In this study we have examined the in vitro effect of taurine on these processes. The migratory potential was assessed using gap closure assay. As shown in Fig. 2, taurine significantly suppressed the migratory movement of human prostate cancer cells, LNCaP at 48 h and PC-3 at 8 h, respectively.

Meanwhile, there is no scientific report for PSA and migration-related genes. Therefore, we also elucidated the effect of taurine on the expression of PSA and migration-related genes such as MMP-9, TIMP-1, TIMP-2, and VEGF. Prostate cancer can increase the amount of PSA released into the blood stream. Notably, PSA present in the extracellular fluid, surrounding prostate epithelial cells, has been reported to be enzymatically active, suggesting that its proteolytic activity plays a role in the physiopathology of prostate cancer (Tomao et al. 2014; Denmeade et al. 2001). MMPs are essential for extracellular matrix remodelling and may contribute to the development of endometriosis (Osteen et al. 2003). It is known that MMP-2 and MMP-9 play important roles in the ectopic adhesion, invasion, and implantation and neovascularisation of the endometrium (Chen et al. 2009). Firstly, we conducted gap closure assay, and found that taurine significantly suppressed the movement of androgen-dependent human prostate cancer cell (Fig. 2). Western blot for the estimation of protein expression of PSA, MMP-2, MMP-9, TIMP-1, TIMP-2, and VEGF was also performed. As shown in Fig. 3, we proved taurine attenuated PSA, MMP-9, and VEGF. However we didn't obtain any evidence of the effect of taurine on MMP-2 protein level in LNCaP and PC-3 cells (data not shown).

There are several reports describing MMP-9 and its specific inhibitor, TIMPs, that are closely correlated with physiological and pathological processes by degradation and accumulation of the ECM (Roderfeld et al. 2007; Goldberg et al. 1989). With the exception of neutrophil granulocytes, MMP-9 is usually secreted together with variable amounts of its specific inhibitor, TIMP-1 and TIMP-2 (Van den Steen et al. 2002). Interestingly, TIMP-1 has been found not only in separated localization, but also in co-localization with pro-MMP-9 in neutrophil organelles, which resemble secretory vesicles (Price et al. 2000). Accordingly, we ascertained the protein level of TIMP-1 and TIMP-2. Figure 3a, d, e show taurine enhance the protein level of TIMP-1 and TIMP-2 in a dose-dependent fashion. Meanwhile, VEGF

induces endothelial cell proliferation, promotes cell migration, and inhibits apoptosis (Neufeld et al. 1999). Accordingly, the VEGF expression was also assessed by Western blot. Figure 3e–g showed that taurine suppressed the expression of VEGF, as well as the expression of PSA which is a marker for prostate cancer.

These results of the present study suggest that taurine has a beneficial effect on the cell death and the expression of PSA, MMP-9, TIMP-1, and TIMP-2 in LNCaP, androgen-dependent human prostate cancer cells.

5 Conclusion

In the present study, taurine improved the apoptosis, the expression of PSA and modulated migration related genes. Although the mechanism for inducing apoptosis and PSA in tumor cells needs further investigation, taurine presents a potential chemotherapy agent.

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Part III
Taurine and Diabetes

The Quest for an Animal Model of Diabetic Nephropathy and the Role of Taurine Deficiency

Xiaobin Han, Takashi Ito, Junichi Azuma, Stephen W. Schaffer, and Russell W. Chesney

Abbreviations

BUN	Blood urea nitrogen
DN	Diabetic nephropathy
GBM	Glomerular basement membrane
SMA	Smooth muscle actin
STZ	Streptozotocin
WT	Wild-type

1 Introduction

Human diabetic nephropathy (DN) is a substantial health problem and is the main cause for renal dialysis in developed countries. In order to undertake a mechanistic approach to DN, a suitable animal model could aid progress. To this end, in 2001, the animal models of the Diabetic Complications Consortium were created

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to develop animal models. These, in turn, could be used to understand pathogenesis, enhance therapeutic approaches and define preventive strategies. While some progress has been achieved in model building, strain analyses, genotype relationships and pathologic processes (Brosius et al. 2009; Gurley et al. 2010; Nakagawa et al. 2007; Qi et al. 2005; Schlondorff 2010), the ideal model has not been found. While advances have been made, a new approach is clearly needed and a probing of specific factors that incite or predict progression of kidney damage may provide new insights.

One feature described in humans with diabetes is taurine deficiency (Franconi et al. 1995; Merheb et al. 2007). Moreover, it has been suggested that taurine supplements can forestall the progression of DN (Hansen 2001). In terms of taurine interactions, DN has been extensively studied (Chesney et al. 2010; Han and Chesney 2012). Most recent studies have addressed potential mechanisms of renal protection by taurine without demonstrating that taurine deficiency promotes a model of DN. One hypothesis is that taurine can ameliorate diabetes-induced oxidative stress. Of several antioxidant enzymes evaluated, such as superoxide dismutase and glutathione peroxidase, heme oxygenase 1 (HO-1) was increased 16-fold in the glomeruli of diabetic rats (Koya et al. 2003). Taurine supplementation reduced HO-1 expression and decreased proteinuria and hypertension. Certain disease-related glomerular pathologic alterations were ameliorated.

When renal tubular epithelial cells were grown in culture with an elevated glucose medium, cellular growth was inhibited and cell hypertrophy occurred (Huang et al. 2007). Addition of taurine to medium blocked Raf-1, p42/p44 mitogen activated protein kinase (MAPK), Janus kinase 2 (JAK2) and signal transducer and activator of transcription 1 (STAT1) and STAT 3 induced by a high glucose milieu (Huang et al. 2007). Taurine addition ameliorated the high glucose-related stimulation of fibronectin and type IV collagen synthesis, increased cyclin D1/cdk^4 and suppressed p21Waf 1/Cip1 and p27(Kip1). In essence taurine reduces cell size, cellular hypertrophy index and protein values for reactive advanced glycation products, Kip1, collagen IV and fibronectin. Hence, taurine may possess antifibrotic activity due to its inactivation of Raf-1/ERK following AEG-induced hypertrophy.

Nodular sclerosis arises from mesangiolysis and detachment of endothelial cells from the glomerular basement membrane (GBM). Certain mononuclear cells, such as T lymphocytes and macrophages, invade the interstitium of the nephron. Finally, there is hyalinosis and dilatation of the efferent arteriole (Bakris 2014; Tervaert et al. 2010).

Another model, the streptozotocin (STZ)-treated rat, had reduced histologic evidence of nephropathy after taurine supplements, and reduced blood urea nitrogen (BUN), serum creatinine and renal malondialdehyde (Wang et al. 2008).

Based upon this body of evidence, we theorized that an animal model that has both the clinical aspects of diabetes mellitus and taurine deficiency might recreate human DN. For this reason, we have recently employed STZ to induce diabetes in wild-type (WT) and *TauT* knockout mice to explore this problem.

2 Methods

2.1 *Animals*

A *TauT* knockout colony was established. Inbred male *TauT* null and age-matched wild-type littermates were developed by mating $TauT^{+/-}$ inbred male and female C57BL/6 mice as described (Ito 2008). We then examined four groups of mice. Twenty of each genotype ($TauT^{+/+}$ and $TauT^{-/-}$) were randomly separated into two groups (n = 10 each) and at 8 weeks of age one group of each genotype was injected for five consecutive days with intraperitoneal STZ (50 mg/kg) (Qi et al. 2005). The other groups of mice (n = 10 each) were injected with buffer alone and served as controls.

Mice were declared diabetic if they developed a blood glucose concentration of more than 250 mg/dL. Mice were studied for 20 weeks after establishment of diabetes. Chemical values measured were BUN, serum and urine albumin and creatinine. Evidence for diabetic nephropathy in mice was assessed using validation criteria of the Animal Models of Diabetic Complications Consortium (AMDC; www.amdcc.org). Animal protocols were approved by the Animal Care and Use Committee of the University of Tennessee Health Science Center.

2.2 *Histology*

After fixation in 10 % formalin neutral buffered solution immediately after sacrifice, kidneys were embedded in paraffin. Two-micrometer thick sections were stained with periodic acid-Schuff reagent (PAS) or hematoxylin and eosin (H + E). Immunohistochemistry was performed using an UltraSensitive ABC Rabbit IgG staining kit and following the manufacturer's specifications (ThermoScientific, Rockford, IL). Samples were rehydrated in decreasing ethanol solutions, then immersed in phosphate buffered saline for 10 min followed by quenching in 3 % hydrogen peroxide. Slides were blocked for 30 min in the kit's blocking solution. Primary antibodies to taurine, taurine transporter protein, smooth muscle actin (SMA), CD34, Ki67 and collagen IV were applied to slides and incubated for an hour. Secondary biotinylated antibodies were applied following another PBS wash and incubated for 1 h. Slides were rewashed for 10 min with PBS, then ABC reagent was applied for 30 min. Immunostaining was detected using a Metal Enhanced DAB Substrate Kit (Thermo Scientific, Rockford, IL).

For electron microscopy, kidneys were fixed in 10 % formalin in neutral buffered solution, embedded in epoxy resin and stained with uranyl acetate and lead citrate. Glomerular basement membrane measurements were made using a JEOL 2000EX transmission electron microscope with a high-resolution digital camera. At least 35 different segments of GBM per mouse from four mice per group were measured. The number of podocytes was counted as well. All quantifications were performed in a blinded fashion.

2.3 Statistics

We performed all animal studies twice. The data represent the mean ± SE of at least four mice. One-way ANOVA and *t*-tests were used to determine significant differences among the means. Significance was defined as $p < 0.05$.

3 Results

To examine the prevalence or absence of TauT protein, kidney cortex slices were stained with antibodies to TauT protein and taurine per se. In non-diabetic WT mice, high levels of TauT protein were found (Fig. 1). As expected, TauT protein was not found in the cortex of *TauT* null mice. As TauT is the specific membrane transporter for renal cell taurine uptake, it would be anticipated that taurine would be absent in *TauT* null mouse kidney. These results indicated that the knockout mice had the relevant phenotype for the study.

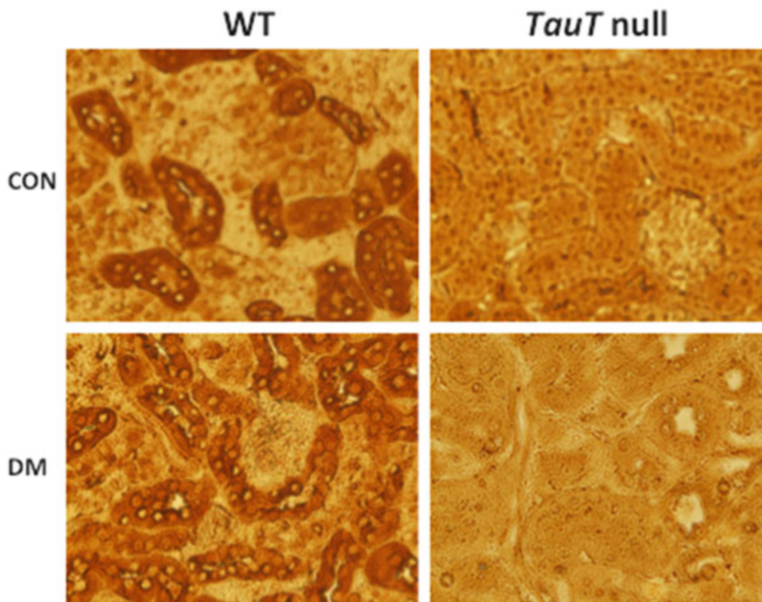

Fig. 1 TauT expression and the presence of taurine in kidney tissue of non-diabetic and diabetic wild-type (WT) and *TauT* null mice. The *brown* color represents both TauT protein. *Con* control, *DM* diabetes mellitus. Magnification: 200×

3.1 Features of Diabetic Mice

Although all diabetic mice were hyperglycemic and non-diabetic mice were normoglycemic when evaluated in the fasting state, *TauT* deletion did not alter glucose values as compared to wild type. Five months post-induction of diabetes, mice from both wild-type and TauT null demonstrated significant weight loss, increased urine albumin, and increased BUN values as compared to controls. Renal weights were significantly increased in diabetic *TauT* null mice 5 months post-induction of diabetes. Notably, kidney weight was nearly doubled in *TauT* null mice 5 months post-induction while body weight was reduced by roughly 30 %. Twenty-four hour urine volume was higher in diabetic WT, but reduced in *TauT* null animals. This latter group had the greatest degree of proteinuria, although all diabetic genotypes had increased albumin/creatinine ratio as evidence of renal disease (Table 1).

3.2 Morphology of Glomeruli

Both WT and *TauT*-deficient diabetic mice showed mesangial expansion after 5 months (Fig. 2). This represents an indication of early diabetic nephropathy. It was more marked in *TauT* null mice. Nodular lesions and nodular glomerulosclerosis were evident 5 months after diabetes was induced in *TauT* null mice. For the most part the more severely affected and damaged glomeruli were located in the juxtaglomerular region. Arteriosclerosis with hyalinosis of arterioles was also found in *TauT* null mice.

Table 1 General characteristics of wild-type and *TauT* null control and diabetic mice

	Wild-type		$TauT^{-/-}$	
Characteristic	Control	Diabetic	Control	Diabetic
5 months after induction of DM				
Blood glucose (mg/dL)	128 ± 15	369 ± 34*	136 ± 32	396 ± 60*
Body weight (g)	31.9 ± 3.2	27.1 ± 2.3	29.3 ± 3.0	18.4 ± 1.8*
Kidney weight (g)	0.23 ± 0.02	0.25 ± 0.03	0.22 ± 0.02	0.35 ± 0.02*
Kidney wt/body wt (×103)	7.2 ± 0.6	9.3 ± 1.0	7.5 ± 0.6	19.0 ± 1.2**
Urine albumin/creatinine (×10)	3.5 ± 1.1	8.9 ± 2.8*	2.7 ± 1.0	21.2 ± 2.8**
BUN (mg/dL)	12.2 ± 1.8	20.3 ± 2.8*	17.2 ± 2.8	31.7 ± 2.9**
12-h urine (μL)	998 ± 88	1,220 ± 200*	856 ± 83	652 ± 79**

DM diabetes mellitus, *BUN* blood urea nitrogen
*$p < 0.05$ vs. control; **$p < 0.01$ vs. control

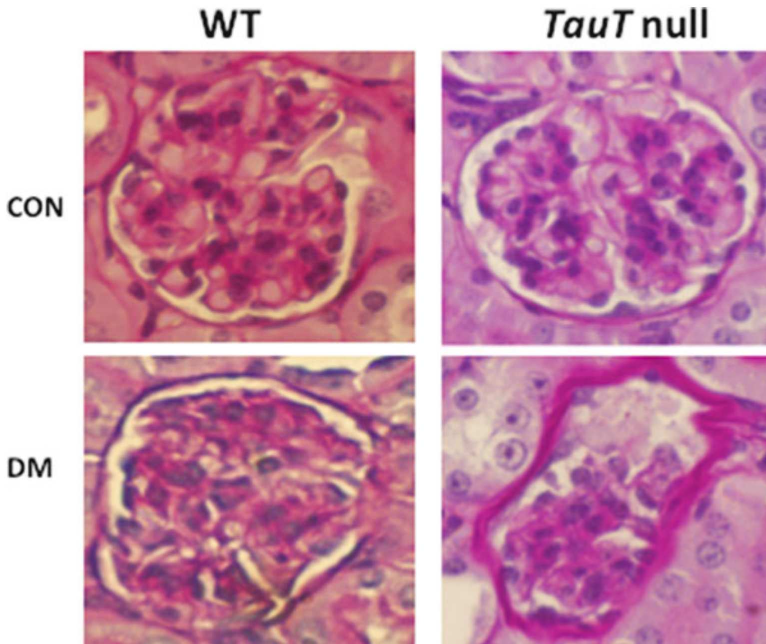

Fig. 2 Histology of glomeruli from wild-type (WT) and *TauT* null mice. Hyalinosis and extension of juxtaglomerular apparatus were seen in diabetic *TauT* null mice at 5 months. *Con* control, *DM* diabetes mellitus. PAS stain; Magnification: 400×

3.3 Histologic Renal Changes in Diabetic TauT-Deficient Mice

A distinctive change in the diabetic *TauT* null mice was in the wall thickness of glomeruli, with dilatation of both afferent and efferent arterioles in or near the glomerulus (Fig. 3). These features closely replicate the alterations in arterioles found in end stage renal disease associated with human diabetic nephropathy. Immunohistochemical staining for SMA revealed that the arterioles were significantly thickened and dilated in *TauT* null diabetic mice at 5 months post-induction relative to controls and WT diabetic mice. At times these changes were associated with necrosis of the glomeruli (so called glomerular drop-out). Although some changes were evident in WT diabetic mice, at 5 months post-induction there was no distinct alteration in glomerular structure, in contrast to diabetic *TauT* null mice. Immunohistochemical staining also revealed that collagen IV was heavily deposited in the juxtaglomerular region in diabetic *TauT* null mice and was associated with mesangial expansion.

Morphologic changes in the endothelium were detected by immunostaining of CD34, a marker of endothelial cells. A generalized increase in the size of endothelial cells was noted in diabetic *TauT* null mice (Fig. 3). This finding was associated

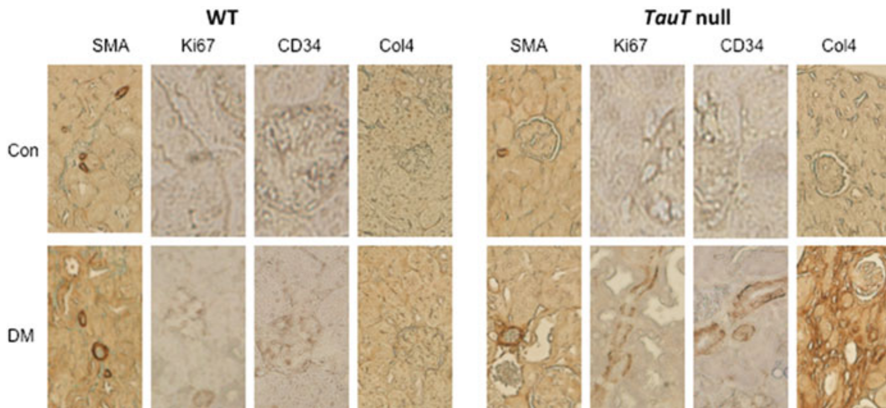

Fig. 3 Histologic renal lesions in diabetic *TauT*-deficient mice. Smooth muscle actin (SMA) in arterioles showed thickened and dilated arterioles in diabetic *TauT* null mice as compared to wild-type (WT) diabetic mice and non-diabetic controls. Immunohistochemical analysis for CD34 and Ki67 (*brown* color) in diabetic $TauT^{+/-}$ and $TauT^{-/-}$ mice at 5 months. Immunohistochemical showing deposition of collagen IV in diabetic *TauT* null mice at 5 months; Magnification: 400×

with enhanced endothelial cell proliferation in the dilated arteriole, as evidenced by staining of Ki67, an endothelial cell proliferation marker. As compared to diabetic WT mice, both CD34 and Ki67 staining was increased in diabetic *TauT* null mice.

Glomerular ultrastructure of 6-month-old mice was assessed by electron microscopy (Fig. 4) as compared to control mice. There was significant thickening of the GBM in diabetic *TauT* null mice (500 ± 45 nm) as compared to controls (200 ± 35 nm). Severe podocyte effacement was evident in diabetic *TauT* null mice.

4 Discussion

Diabetic nephropathy in humans is the primary cause of end stage renal disease, leading to a requirement for dialysis and/or transplantation (USRDS 2013). Hence, the quest for an appropriate renal model that mimics human disease is an important goal. Most models have been incomplete models, and fail to contain all the elements of human DN, including increased mesangial matrix, hyalinosis, fibrous thickening of efferent and afferent arterioles with dilatation and atherosclerosis with progression to Kimmelsteil-Wilson nodules, and increased GBM thickness (Brosius et al. 2009; Leiter and Schile 2013). Certain mouse strains appear to be better platforms for the construction of a model (Franzen et al. 2014), with BALB/c showing the greatest histologic damage in an alloxan induction model. The C57Bl/6 strain, the platform for our model, has not performed as well, at least in WT animals.

Recent studies indicate that both BRKO-Akita and BTBR *ob*/*ob* mice develop more advanced nephropathy than earlier murine models (Hudkins et al. 2010;

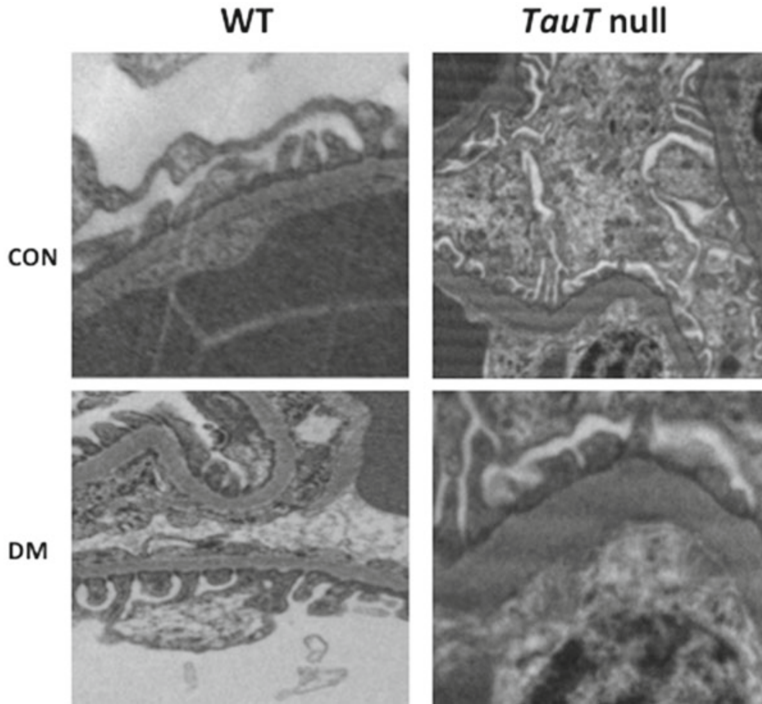

Fig. 4 Electron micrographs of glomeruli in wild-type (WT) and *TauT*-deficient diabetic mice. Con: non-diabetic WT and *TauT*$^{-/-}$ mice with preserved podocyte with foot processes and basement membrane. Diabetic WT kidney with red blood cells in capillary lumen, preserved basement membrane and podocyte with foot processes. Glomeruli from a diabetic *TauT* null mouse with uneven and thickened glomerular basement membrane (GBM), markedly expanded mesangium, and podocyte effacement. Magnification: 10,000×

Kakoki et al. 2010), but these two models do not develop end stage renal disease, as characterized by a more than 50 % decline in GFR during the lifespan of the animal. Another shortcoming is that the BTBR *ob*/*ob* mouse model relies on leptin deficiency, which is not a feature of human diabetes (Hudkins et al. 2010). Diabetic C57Bl/6 and NMRI both developed glomerular hyperfiltration, but neither presented with histological damage, although NMRI developed low-degree proteinuria. Neither diabetic BALB/c nor 129Sv developed pronounced proteinuria. Only BALB/c developed detectable histological damage, suggesting that BALB/c may be suitable when studying the roles of proteinuria and histological alterations for the progression of diabetic nephropathy (Franzen et al. 2014).

Our studies were designed to determine whether taurine status would influence the development of DN in the diabetic mouse. This concept is based upon two lines of evidence: 1) taurine deficiency is evident in diabetic patients (Franconi et al. 1995; Merheb et al. 2007) and 2) taurine excess (or supplementation) is protective against acute kidney injury (Han and Chesney 2012). To wit, could a

lack of taurine sufficiency constitute a potential risk factor for the development of chronic kidney disease in a mouse model? We posited that the *TauT* mouse, made diabetic with STZ, would produce a more robust model of DN more closely resembling human disease.

The features we sought to reproduce include expansion of mesangial cells, thickening of the glomerular basement membrane, and glomerular sclerosis (Tervaert et al. 2010). Other features include arterionephrosclerosis, ischemic changes with accompanying interstitial fibrosis, and tubular atrophy. The glomerular basement membrane is thickened by extracellular matrix accumulation of collagens IV and VI, laminin and fibronectin.

In the present study, we present evidence in *TauT* null animals of mesangial cell expansion, glomerular basement thickening, nodular sclerosis with expansion of the glomerulus beyond Bowman's capsule, and juxtaglomerular tuft sclerosis. Additional typical features of human diabetic nephropathy include atherosclerosis, ischemic changes, glomerular dropout, and interstitial fibrosis. Thus, there is mimicking of the features of human diabetic nephropathy in this model from a renal histologic point of view. Immunohistochemical changes include increased deposition or abundance of SMA, collagen IV, CD34 (as a marker of endothelial injury) and Ki67.

The clinical and phenotypic features of this model include azotemia, persistent hyperglycemia, heavy proteinuria and progressive renal disease. Other aspects include an increased renal weight/body weight ratio in diabetic *TauT* null mice as compared to control animals, as would be expected in a nephrotic animal with mesangial cell expansion and nodular sclerosis.

5 Conclusion

To summarize, the *TauT* null mouse can serve as an excellent platform on which to create a STZ-induced animal model of diabetic nephropathy. This model mimics numerous features of human diabetic nephropathy, including its clinical aspects and prototypic renal histologic findings. Results from this study also strongly suggest that the malfunction of the taurine transporter found in diabetes patients is an important risk factor for developing kidney disease, including diabetic nephropathy. Thus, one can use this model to design relevant studies and gain greater insight into the causes and consequences of human diabetic nephropathy.

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Taurine Can Enhance the Protective Actions of Metformin Against Diabetes-Induced Alterations Adversely Affecting Renal Function

Kashyap G. Pandya, Roshil Budhram, George J. Clark, and Cesar A. Lau-Cam

Abbreviations

INS	Insulin
MET	Metformin
STZ	Streptozotocin
TAU	Taurine

1 Introduction

Diabetic nephropathy, also known as diabetic kidney disease, is a common microvascular complication of type 1 and type 2 diabetes mellitus characterized by microalbuminuria that can progress to persistent albuminuria, progressive decline in the estimated glomerular filtration rate, and hypertension (United States Renal Data System 2013). Additional findings include dyslipidemia, low grade inflammation, insulin resistance (Thorn et al. 2005) and morphological alterations in the glomerulus, basement membrane, mesangial cells, tubules and arterioles (Kashihara et al. 2010).

Diabetic nephropathy is the result of the action and interaction of numerous metabolic and hemodynamic factors on the kidney, contingent to the level of renal microcirculation, having a genetic underlining, and hypertension and hyperglycemia as its most prevalent modifiable factors (Mota et al. 2009). In the setting of mild-to-moderate renal insufficiency, metformin (MET) has emerged as a first-line therapy for people with type 2 diabetes and obesity on account of its ability to improve glucose uptake in adipose tissue and skeletal muscle, decrease hepatic

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glucose production, improve peripheral sensitivity to INS action, and reduce the circulating levels of free fatty acids but without stimulating INS secretion, aggravating hyperinsulinemia, causing hypoglycemia or promoting weight gain (Davidson and Peters 1997; Derosa and Sibilla 2007). In addition MET has demonstrated a significant lowering effect on the plasma total cholesterol, low-density lipoprotein (LDL)-cholesterol and plasma triglycerides (DeFronzo et al. 1991; Wuffelé et al. 2004), and a smaller lowering effect on the ratio of LDL- to high-density lipoprotein (HDL)-cholesterol and on apolipoprotein B concentration (Carlsen et al. 1996). The renoprotective properties of MET in diabetes have been investigated in STZ-treated rats at two different doses (Alhaider et al. 2011). In addition to raising the renal levels of ATP, reduced glutathione (GSH), and ATP/AMP ratio, MET has also been found to reduce reactive oxygen species (ROS) production, to restore the expression of antioxidant genes while inhibiting that of proinflammatory cytokine (tumor necrosis factor-α, interleukin-6) genes in a dose-dependent manner, and to preserve the normal histology of the kidney tissue. However there is also evidence indicating that in the same animal model of diabetes MET was ineffective in attenuating the decreases in catalase and glutathione reductase activities, in total antioxidant status, and in GSH levels brought about by diabetes (Erejuwa et al. 2011).

Taurine (TAU) is a conditional nonprotein amino that has been extensively investigated for its attenuating effects on diabetes-related alterations such as decreased insulin secretion (Kulakowski and Maturo 1984; Tokunaga et al. 1983), hyperglycemia (Kulakowski and Maturo 1984; Tan et al. 2007), hyperlipidemia (Goodman and Shihabi 1990; Tan et al. 2007), lipid peroxidation (LPO) (Goodman and Shihabi 1990; Tan et al. 2007; Trachtman et al. 1995), and formation of advanced glycated end (AGE) products (Trachtman et al. 1995) in spontaneous and pharmacologically-induced animal models of diabetes. In addition to improving hyperglycemia, insulin secretion and sensitivity, and dyslipidemia, TAU has also shown the ability to attenuate oxidative stress, protein and hemoglobin glycation, and LDL oxidation, and to protect against the manifestations of atherosclerosis, cardiomyopathy, retinopathy, neuropathy, nephropathy and vascular dysfunction in different animal models of type 1 and of type 2 diabetes (Ito et al. 2012). When used in humans with or without diabetes, however, the results have led to mixed results, with some studies demonstrating positive effects and others reporting failure. With the possible exception of its consistent normalizing effect on endothelial dysfunction, the effects of TAU on hyperglycemia, insulin secretion and resistance, and microalbuminuria have been conflicting (Ito et al. 2012). Several studies have established the beneficial effects of TAU in the diabetic kidney. For example, recently TAU was shown to alleviate the progression of diabetic nephropathy in a rat model of diabetes by virtue of its protective action against the metabolic alterations, fibrosis and oxidative stress caused by this disease in the kidney (Koh et al. 2014). In this laboratory a 6 week daily oral treatment of STZ-diabetic rats with TAU was found to significantly reduce the hyperglycemia, dyslipidemia, elevation of the blood glycated hemoglobin (HbA_{1c}) level, oxidative stress in erythrocytes and kidney, and changes in biochemical indices of renal dysfunction as well as to minimize histological changes in the diabetic kidney (Budhram et al. 2013; Pandya et al. 2013).

Based on the understanding that has developed over the years on the pathophysiologic mechanisms responsible for the development of diabetic nephropathy, therapeutic approaches for the prevention of this type of diabetic complication have been aimed at maintaining a tight control on the blood glucose, on the blood pressure, and at lowering albuminuria (Stanton 2011). An additional potential target is oxidative stress since it can serve as a stimulus for signaling pathways mediating cell dysfunction and apoptotic cell death, for protein modification by glycation, and for the formation of the profibrotic transforming growth factor-1β (TGF-1β). Indeed, activation of the renin-angiotensin system driven by hyperglycemia and mechanical stress leads to the release of angiotensin II which, together with high glucose, may stimulate the influx of proinflammatory cells capable of releasing TGF-β1, a cytokine found to promote interstitial fibrosis and mesangial and tubular hypertrophy by inhibiting extracellular matrix degradation and stimulating matrix synthesis (Więcek et al. 2003). In experimental diabetic nephropathy there is also an increase in vascular oxidative stress and in synthesis of damaging ROS, produced in part as a result of the activation of nicotinamide dinucleotide phosphate reduced (NAD(P)H) oxidase by angiotensin II, with additional contributions made by xanthine oxidoreductase under the influence of hyperuricemia and by the oxidation of advanced glycation end products, formed by the nonenzymatic binding of the aldehyde group of glucose with free amino groups in renal proteins, and which can accumulate in renal tissues and generate ROS (Kashihara et al. 2010).

The present study was undertaken to compare MET and TAU for their ability to attenuate metabolic changes, renal and plasma oxidative stress, and renal function impairment as a result of diabetes in a streptozotocin-based rat model of diabetes. An additional objective was to determine whether a combined treatment with MET plus TAU can offer any advantage over the individual treatments.

2 Methods

2.1 Animals and Treatments

Male Sprague-Dawley rats, 225–250 g, acclimated for 1 week in a room maintained at a constant humidity and temperature (23 ± 1 °C) and a normal 12 h light–12 h dark cycle room, and assigned to groups of 6 were used in the experiments. The rats had free access to a commercial rodent diet and filtered tap water. The study was approved by the Institutional Animal Care and Use Committee of St. John's University, Jamaica, NY, and the animals were cared in accordance with guidelines established by the United States Department of Agriculture. Diabetes was induced with a single 60 mg/kg intraperitoneal dose of streptozotocin (STZ) in 10 mM citrate buffer pH 4.5. Starting on day 15 and continuing for the next 41 days, separate groups of diabetic rats received a 2.4 mM/kg daily dose of MET, TAU or MET plus TAU by oral gavage or 4 units/kg/day dose of 70 % NPH insulin (INS) suspension by the subcutaneous route. Rats in the control group received only 10 mM

citrate buffer pH 4.5 in a volume equal to that of the STZ solution, and rats in the diabetic control group received only STZ on day 1.

2.2 Samples and Assays

The development and course of the diabetes was monitored on a weekly basis by measuring the concentration of blood glucose on a drop of tail vein blood with the help of a blood glucose meter (TRUEtrack™) and test strips (Nipro Diagnostics, Fort Lauderdale, FL). Only those rats exhibiting a blood glucose level >300 mg/dL were used in the study. On day 56, all the animals were placed in metabolic cages, one per cage, to obtain 24 h urine samples for biochemical testing, after which they were sacrificed by decapitation to collect blood samples in heparinized tubes and to remove the kidneys by the freeze-clamp technique of Wollenberger et al. (1960). The blood samples were divided into two portions, one portion was used to assay the glycated hemoglobin (HbA_{1c}) content and the other portion was processed for its plasma fraction, which was used for the assay of indices of metabolic impairment, oxidative stress and renal function and for ascertaining the occurrence of nephropathy. The kidneys were homogenized with Tris buffer pH 7.0 containing 1 mg of phenylmethylsulfonyl fluoride (1:20 ratio) over ice, and the resulting suspensions were centrifuged at 12,000 rpm and 4 °C for 30 min, to obtain clear supernatants suitable for the determination of indices of oxidative stress. The urine samples were used to evaluate glomerular and tubular status.

The plasma glucose content was measured using a commercially available colorimetric kit (Procedure No. 510 from Sigma-Aldrich, St. Louis, MO), which is based on the method of Raabo and Terkildsen (1960). The results were expressed in mg/dL. The concentration of INS in the plasma was measured by means of a commercial assay kit (Insulin ELISA kit, Calbiotech Inc., Spring Valley, CA). The results were expressed in μIU/mL. The concentration of blood HbA_{1c} was measured using a commercial optimized ion-exchange resin procedure (Glycohemoglobin Test, Stanbio Laboratory, Boerne, TX). The results were expressed as a percentage of the total hemoglobin content. The contents of plasma and urine creatinine were measured with a commercially available colorimetric assay kit (Kinetic Creatinine LiquiColor® Test, Stanbio Laboratory, Boerne, TX). The results were expressed in mg/dL. The contents of plasma and urine total protein were measured with a colorimetric assay kit based on the Biuret reaction (Protein, Total LiquiColor® Test, Stanbio Laboratory, Boerne, TX). The results were expressed in g/dL. The concentration of Na^+ in the plasma and urine was measured colorimetrically with a commercially available assay kit based on its reaction with a reagent containing uranyl acetate-zinc acetate (Sodium Test, Stanbio Laboratory, Boerne, TX). The results were expressed in mmol/L. The concentrations of K^+ in the plasma and urine were measured by a turbidimetric assay method after reaction with alkaline sodium tetraphenylboron (Potassium Test, Stanbio Laboratory, Boerne, TX). The results were expressed in mmol/L. The concentration of MDA in plasma and kidney was

measured as thiobarbituric acid reactive substances (TBARS) by the end point assay method of Buege and Aust (1978). The results were expressed in nmol/mL of plasma or nmol/g of tissue. Both the kidney homogenate and plasma levels of GSH and GSSG were measured fluorometrically by the method of Hissin and Hilf (1976), which is based on the reaction of GSH with *ortho*-phthalaldehdye (OPT) at pH 8.0 and of GSSG with OPT at pH 12.0. Prior to the measurement of GSSG, any interfering GSH is complexed with N-ethyl maleimide according to the method of Guntherberg and Rost (1966) to prevent its interfering effect on the measurement of GSSG. The concentrations of GSH and GSSG were expressed as nmol/mL of plasma or nmol/g of tissue. The plasma level of TGF-β1 was measured using a commercially available ELISA kit (Invitrogen™ TGF-β1 Multispecies ELISA kit, Life Technologies, Grand Island, NY). The results were expressed in pg/mL.

2.3 Statistical Analysis of the Data

The results, reported as mean ± standard error of the mean (SEM) for $n=6$ rats, were analyzed for statistical significance by unpaired Student's t-test, followed by one-way analysis of variance (ANOVA) and Tukey's *post hoc* test. Intergroup differences were considered to be significant when p was ≤ 0.05.

3 Results

3.1 Metabolic Changes

At the end of 1 week the blood glucose of diabetic rats had risen by more than four-fold over the control value. Although the blood glucose level remained elevated in the ensuing weeks, at the end of 8 weeks it had fallen to a value that was 3.9-fold above the control value (Fig. 1). A daily treatment with MET was very effective in reducing the hyperglycemic state in a consistent and significant manner, with the 8 week value representing a 40 % decrease ($p<0.01$). TAU, on the other, was only significantly effective ($p<0.05$ vs. diabetes) during the last 2 weeks and with only about one-half the potency of MET. A combined treatment with MET plus TAU resulted in an effect indistinguishable from that by MEt alone. For the first 2 weeks of treatment, INS was as effective as MET in controlling hyperglycemia, but over the following 4 weeks it gradually brought the blood glucose to the normal value. The result obtained with blood glucose samples were found to be in very close agreement (≤ 5 % difference) with the values recorded using plasma values (Fig. 1).

The plasma INS level of diabetic rats was found markedly reduced (by 76 %, $p<0.001$) in diabetic rats compared to the control value (Fig. 2). Both MET and TAU were able to attenuate the decrease in INS secretion, with the former compound appearing twice as potent as the latter one (26 %, $p<0.05$, and 52 %, $p<0.001$,

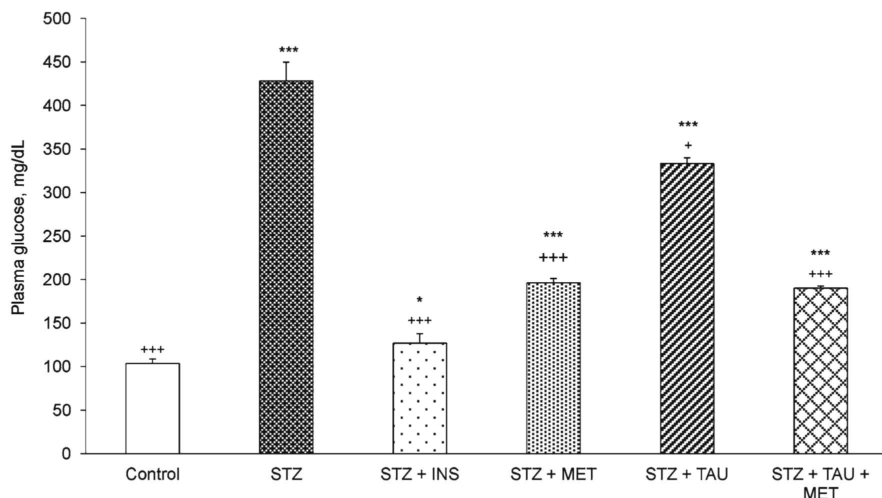

Fig. 1 The effect of treating diabetic (STZ) rats with INS, MET, TAU and MET-TAU, starting 15 days after a STZ dose and continuing for the next 41 days, on the diabetic plasma glucose level. Differences were significant vs. Control at $^{*}p<0.05$ and $^{***}p<0.001$; and vs. STZ at $^{+}p<0.05$ and $^{+++}p<0.001$.

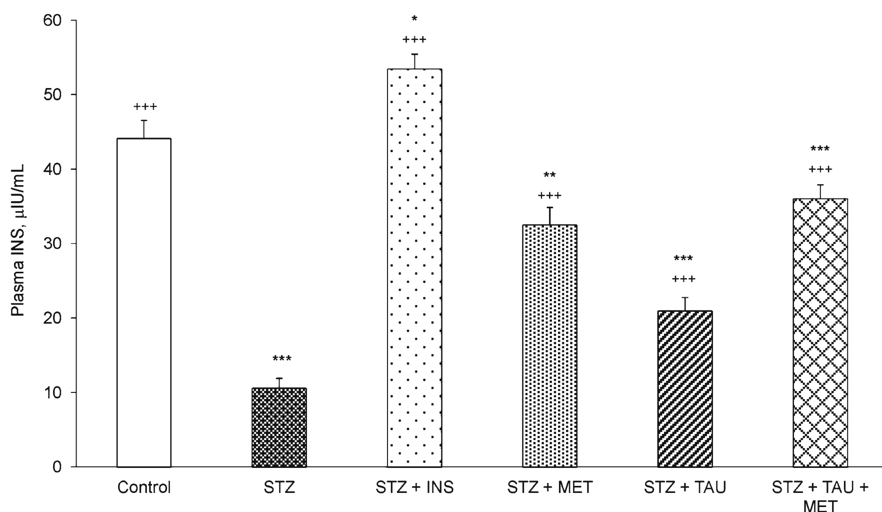

Fig. 2 The effect of treating diabetic (STZ) rats with INS, MET, TAU and MET-TAU, starting 15 days after a STZ dose and continuing for the next 41 days, on the diabetic plasma INS level. Differences were significant vs. Control at $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$; and vs. STZ at $^{+++}p<0.001$.

decreases, respectively). This protection was enhanced when MET plus TAU were given concurrently (only 18 % decrease, $p<0.05$). INS of exogenous origin raised the circulating level by ~20 % above the baseline value ($p<0.05$ vs. control) (Fig. 2).

In comparison to control rats, diabetic ones exhibited a threefold increase in blood HbA_{1c} levels ($p<0.001$) (Fig. 3). A treatment with MET reduced the HbA_{1c} concentration to a value not significantly different from the control value (only 13 % increase), an effect that was maintained in the presence of TAU (11 % increase). Both TAU and INS were also able to reduce the diabetic levels of HbA_{1c} (increases of 59 % and 52 %, respectively, $p<0.001$ vs. control) but to a lesser extent than MET (Fig. 3).

Diabetes raised the circulating levels of both cholesterol and triglycerides to values that were significantly higher than the corresponding control values (by 66 % and 155 %, respectively, both at $p<0.001$) (Fig. 4). A treatment with MET lowered the diabetic values of these lipids by 30 % and 45 %, respectively ($p\leq0.01$ vs. diabetes); and one with TAU provided a protection equivalent to that by MET (decreases of 29 % and 49 %, respectively, $p\leq0.01$ vs. diabetes). On the other hand, a combined treatment with MET plus TAU led to an insignificant enhancement of the hypolipidemic effect achieved with the individual treatments (decreases of 38 % and 54 %, $p\leq0.01$ vs. diabetes). INS was as protective as MET or TAU on the diabetic plasma triglycerides level (28 % decrease, $p<0.01$) and more potent than these compounds on the diabetic plasma cholesterol level (64 % decrease, $p<0.001$) (Fig. 4).

3.2 *Oxidative Stress*

Evidence of a state of oxidative stress in diabetes was obtained by measuring the extent of lipid peroxidation (LPO) and the changes in the ratio of GSH/GSSG in the plasma and liver. Based on the levels of MDA, it was apparent that diabetes

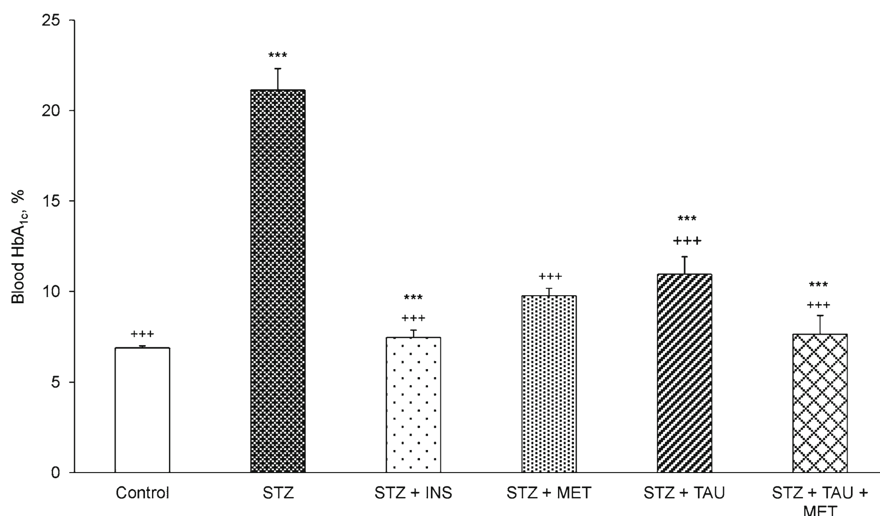

Fig. 3 The effect of treating diabetic (STZ) rats with INS, MET, TAU and MET-TAU, starting 15 days after a STZ dose and continuing for the next 41 days, on the diabetic blood HbA_{1c} level. Differences were significant vs. Control at $^{***}p<0.001$; and vs. STZ at $^{+++}p<0.001$.

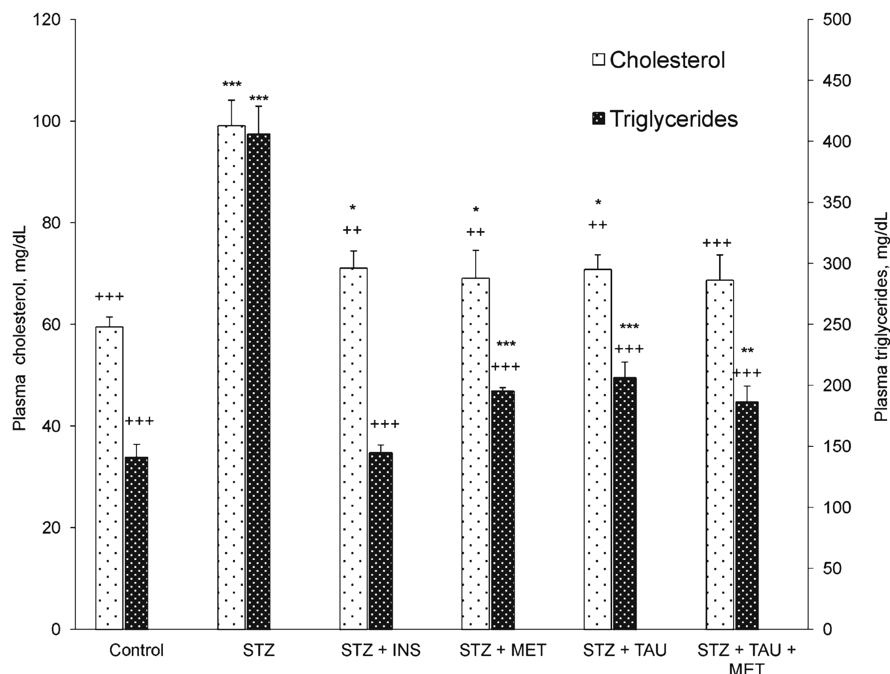

Fig. 4 The effect of treating diabetic (STZ) rats with INS, MET, TAU and MET-TAU, starting 15 days after a STZ dose and continuing for the next 41 days, on the diabetic plasma cholesterol and triglycerides levels. Differences were significant vs. Control at $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$; and vs. STZ at $^{++}p<0.01$ and $^{+++}p<0.001$.

promoted a LPO that reached equivalent extents (41 % and 42 % increases, respectively, $p<0.01$ vs. control) in the plasma and kidney (Fig. 5). A treatment with MET, TAU or their combination was found to virtually abolish MDA formation (only 1–6 % increases). The same degree of protection was attained with an INS treatment (Fig. 5). Diabetes reduced the GSH level in the plasma (by 46 %, $p<0.001$) and kidney (by 34 %, $p<0.01$) together with the corresponding GSH/GSSG ratios (by 75 % in the plasma, by 71 % in the kidney, both at $p<0.001$ vs. controls) (Fig. 6). These changes were effectively counteracted by all the treatment agents ($p\leq 0.01$), with TAU and MET-TAU providing the greatest protection, followed by INS and MET.

3.3 Renal Function and Nephropathy

The renal function of diabetic rats was compared with that of normal rats on the basis of changes in urine volume production, degree of proteinuria, and changes in plasma creatinine, urea nitrogen, Na^+, K^+ and TGF-β1 levels (Figs. 7, 8, 9, 10, and 11).

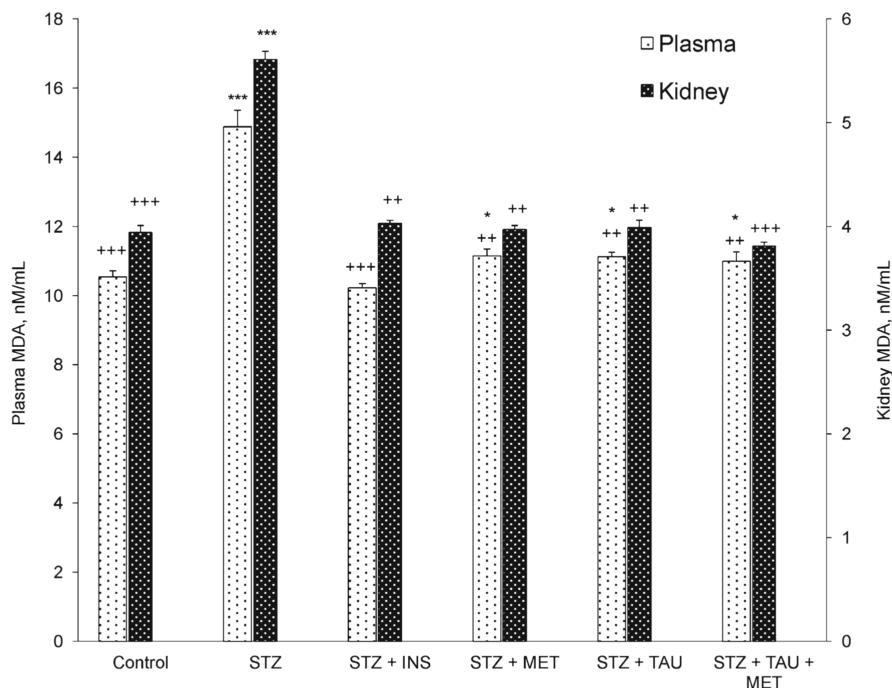

Fig. 5 The effect of treating diabetic (STZ) rats with INS, MET, TAU and MET-TAU, starting 15 days after a STZ dose and continuing for the next 41 days, on the diabetic plasma and kidney MDA levels. Differences were significant vs. Control at $^{*}p<0.05$ and $^{***}p<0.001$; and vs. STZ at $^{++}p<0.01$ and $^{+++}p<0.001$.

Diabetic rats showed a 5.7-fold increase in the urine output (60 mL/day) over that of normal rats. INS was very effective in reducing this output (only threefold increase), an effect that was shared, although to a lesser degree, by MET, TAU and MET-TAU (4.7-fold, 4.9-fold and 4.6-fold increases, respectively, $p<0.05$ vs. diabetes) (Fig. 7). The massive excretion of proteins in the urine seen in diabetic rats (>150 % increase) was drastically reduced by all the treatment agents ($p\leq 0.01$ vs. diabetes), with INS showing the greatest effect (by 60 %), followed by MET (by 49 %) and TAU (by 40 %). The effect of a combined treatment with MET-TAU was intermediate to that seen with the individual compounds (46 % decrease) (Fig. 8). Diabetic rats exhibited significantly higher levels of plasma creatinine and urea nitrogen than control rats (by >330 % and >150 %, respectively, $p<0.001$) (Fig. 9). MET and TAU were able to reduce these changes significantly, with TAU providing a slightly greater protective effect (54 % and 35 % reductions, respectively) than MET (45 % and 22 % reductions, respectively). Relative to the individual treatments, one with TAU plus MET led to a greater effect in the case of the plasma urea nitrogen (52 % reduction) but not in the case of the plasma creatinine (43 % reduction). INS was as effective as TAU in reducing the diabetic plasma creatinine (58 % less, $p<0.001$) and as MET-TAU on the plasma urea nitrogen (49 % less, $p<0.001$) (Fig. 9). Diabetes also affected the plasma levels of Na^{+} and K^{+}, which were found

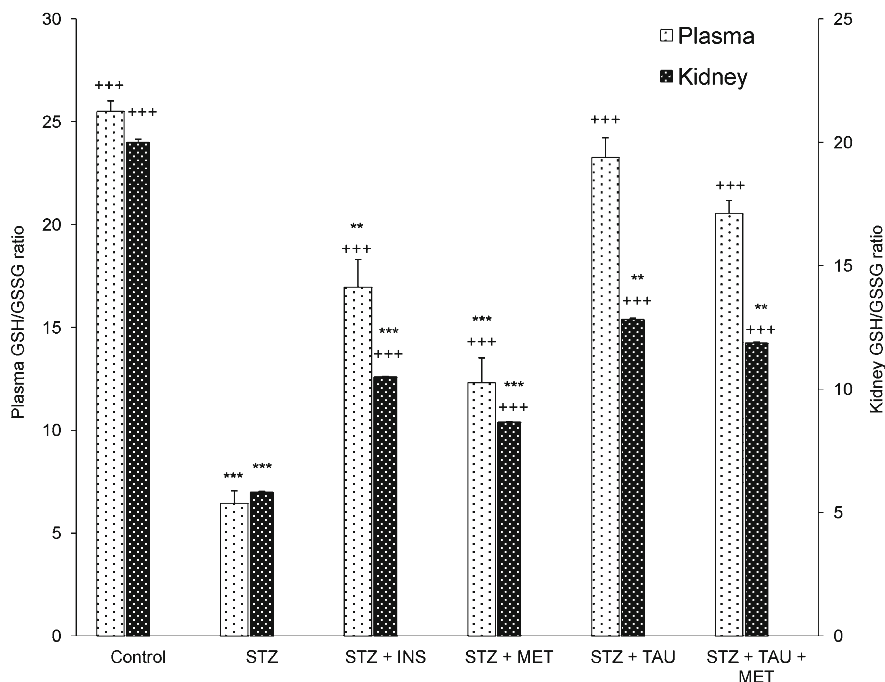

Fig. 6 The effect of treating diabetic (STZ) rats with INS, MET, TAU and MET-TAU, starting 15 days after a STZ dose and continuing for the next 41 days, on the diabetic plasma and kidney GSH/GSSG ratios. Differences were significant vs. Control at $^{**}p<0.01$ and $^{***}p<0.001$; and vs. STZ at $^{+++}p<0.001$.

significantly higher than in control rats (by 35 % and 56 %, respectively, $p \leq 0.01$) (Fig. 10). In contrast, treatments with MET, TAU, MET-TAU and INS kept the diabetic plasma levels to within 2–11 % of the control values. The potential for diabetes-induced nephropathy was investigated by measuring the plasma levels of TGF-β1. As depicted in Fig. 11, diabetic rats showed a 12.4-fold increase compared to control rats ($p<0.001$). A treatment of diabetic rats with MET, TAU or MET-TAU kept the plasma TGF-β1 to concentrations below fourfold over the control value, with MET-TAU providing a greater protection (3.25-fold increase) than either MET (3.83-fold increase) or TAU (3.85-fold increase). Providing INS on a daily basis reduced the elevation to only about 1.3-fold over the control value.

4 Discussion

Diabetic kidney disease is a glomerulopathy distinguished by characteristic structural and functional changes and by typical clinical manifestations. In addition to structural changes at the glomeruli and renal tubules, interstitium and arterioles, especially in later stages of the disease (Fioretto and Mauer 2007), there are also

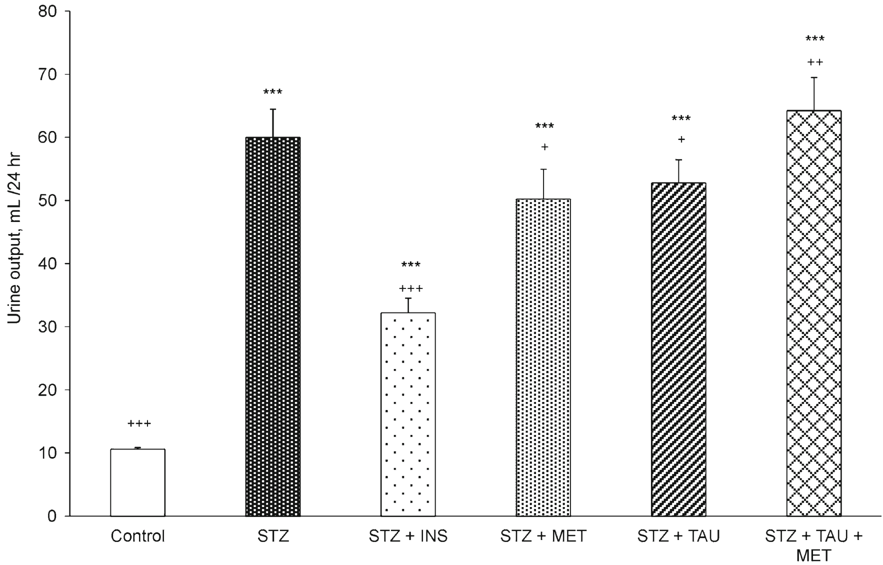

Fig. 7 The effect of treating diabetic (STZ) rats with INS, MET, TAU and MET-TAU, starting 15 days after a STZ dose and continuing for the next 41 days, on the diabetic urinary output. Differences were significant vs. Control at $^{***}p<0.001$; and vs. STZ at $^{+}p<0.05$, $^{++}p<0.01$ and $^{+++}p<0.001$.

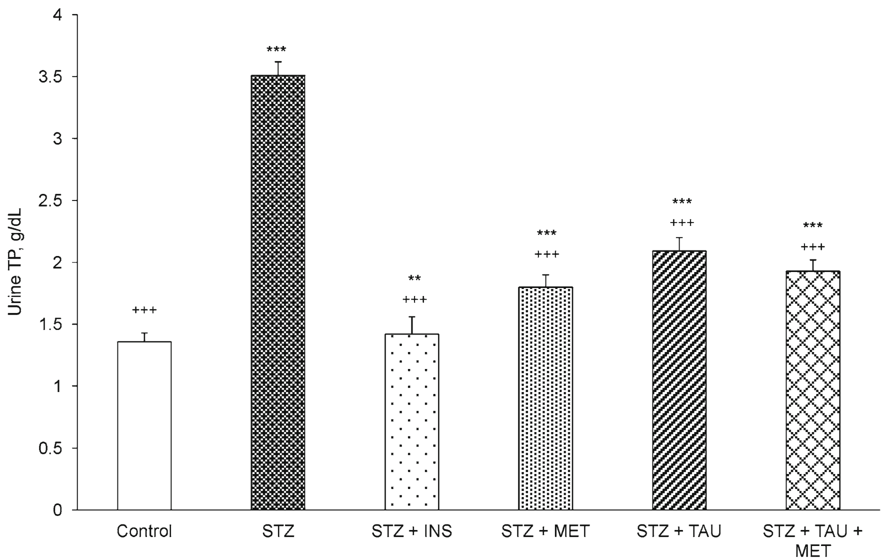

Fig. 8 The effect of treating diabetic (STZ) rats with INS, MET, TAU and MET-TAU, starting 15 days after a STZ dose and continuing for the next 41 days, on the diabetic urine TP level. Differences were significant vs. Control at $^{**}p<0.01$ and $^{***}p<0.001$; and vs. STZ at $^{+++}p<0.001$.

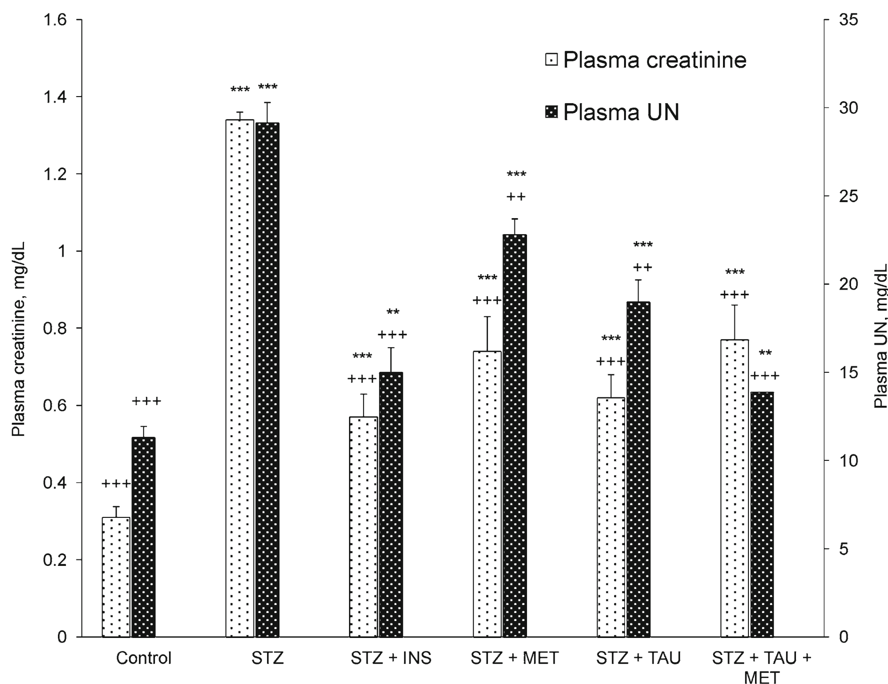

Fig. 9 The effect of treating diabetic (STZ) rats with INS, MET, TAU and MET-TAU, starting 15 days after a STZ dose and continuing for the next 41 days, on the diabetic plasma creatinine and urea nitrogen levels. Differences were significant vs. Control at $^{**}p<0.01$ and $^{***}p<0.001$; and vs. STZ at $^{++}p<0.01$ and $^{+++}p<0.001$.

glomerular functional changes such as glomerular hyperfiltration and hyperperfusion (Caramori and Mauer 2003; Dronavalli et al. 2008). Important clinical manifestations include varying degrees of proteinuria, a persistent hyperglycemia, increased HbA_{1c} values, dyslipidemia, hypertension, and reduced glomerular filtration rate (Bojesting et al. 1994; Caramori et al. 2003; Ismail et al. 1999).

This study has assessed the actions of MET and TAU, singly and in combination, on several major risk factors of diabetic kidney disease and on the consequences of these actions on the ensuing renal function. In this context, hyperglycemia appears to be an important contributing factor in the development of proteinuria in type 2 diabetes and a hastening factor for the occurrence and progress of diabetes-related nephropathy (Ismail et al. 1999). In this study, the daily oral administration of either MET or TAU was found to bring about a significant attenuation of the diabetic hyperglycemia, with MET providing twice as much protection as TAU. The possibility that MET might be acting in part by a mechanism analogous with to that of TAU was suggested by the complete lack of effect of TAU in modifying the hypoglycemic potency of MET. MET was also able to elevate the circulating diabetic levels of INS significantly, to a value that was more than 1.5-fold higher than that by TAU. MET could be exerting its increasing effect by a mechanism similar to that of

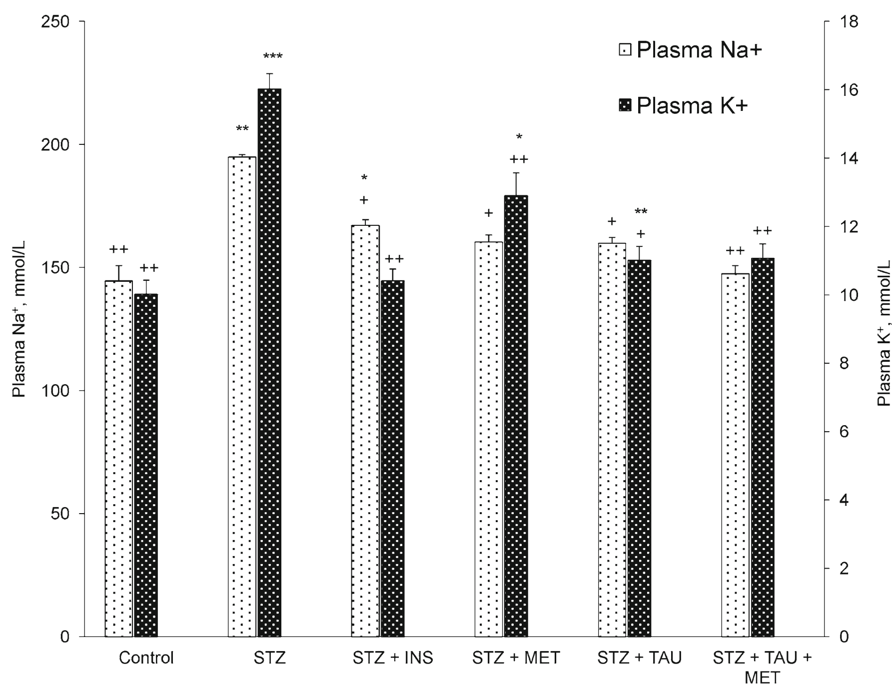

Fig. 10 The effect of treating diabetic (STZ) rats with INS, MET, TAU and MET-TAU, starting 15 days after a STZ dose and continuing for the next 41 days, on the diabetic plasma Na^+ and K^+ levels. Differences were significant vs. Control at $^*p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$; and vs. STZ at $^+p<0.05$ and $^{++}p<0.001$.

TAU since the effect of a combined treatment with MET-TAU was not very different from that with MET alone. In this respect, both MET and TAU could be attenuating diabetic hyperglycemia by improving INS sensitivity through an effect on lipid metabolism (Fullerton et al. 2013; Oprescu et al. 2007). The effectiveness of both MET and TAU in improving glycemic control was determined on the basis of changes in whole blood HbA_{1c} levels. MET was able to reduce the diabetic HbA_{1c} to a value that was not significantly different from the control value but significantly lower than in the presence of TAU. On the other hand, adding TAU to a treatment with MET had no effect on the activity, of MET on HbA_{1c}. Except for its effect on the HbA_{1c} level, which was close to that seen with TAU, INS was found to normalize the changes in blood glucose and in plasma INS.

Prospective studies have verified a link between disturbed circulating lipids and the development and progression of albuminuria and microvascular renal disease among the diabetic population although the underlying mechanisms are still a matter of debate (Rosario and Prabhakar 2006; Thomas et al. 2006). Hence, the control of hyperlipidemia is one of the cornerstones in the treatment of type 1 diabetes. The present results suggest that MET and TAU can lower the hypercholesterolemia and hypertriglyceridemia of diabetes to about the same extent. Moreover, supplementing

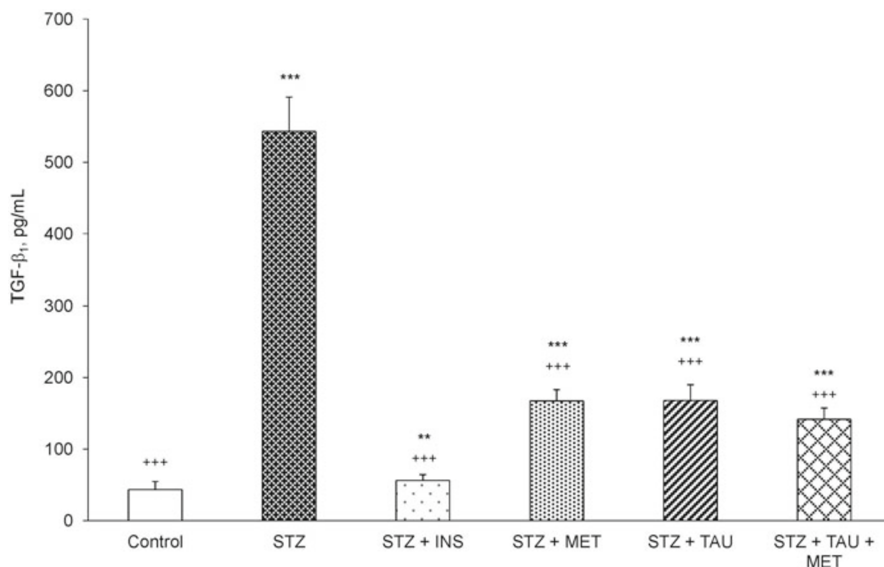

Fig. 11 The effect of treating diabetic (STZ) rats with INS, MET, TAU and MET-TAU, starting 15 days after a STZ dose and continuing for the next 41 days, on the plasma TGF-β1 level. Differences were significant vs. Control at $^{**}p<0.01$ and $^{***}p<0.001$; and vs. STZ at $^{+++}p<0.001$.

MET with TAU led to a small improvement in cholesterol control and to a significant improvement in triglycerides control. In contrast, a treatment with INS led to a hypocholesterolemic effect equivalent to that of TAU and to hypotriglyceridemic effect greater than that of either MET or TAU. The lowering effect of MET on triglycerides levels may be multifactorial since it may arise from an ability to reduce the expression of the enzyme fatty acid synthase or the activity of acetyl CoA carboxylase activity, to induce fatty acid oxidation, to suppress the expression of lipogenic factors in the liver (Zhou et al. 2001), and to increase kidney and liver lipoprotein lipase activity and the hepatic secretion of very low-density lipoprotein (VLDL)-triglycerides (Anurag and Anuradha 2002). In addition, MET may lower cholesterol levels by inhibiting the activity of hydroxymethylglutaryl coenzyme A reductase activity, the key enzyme for cholesterol biosynthesis (Koren-Kluzer et al. 2013). The lipid lowering actions of TAU have been demonstrated both in the serum and in the liver of rodents fed cholesterol rich- and cholesterol-free diets. In the rat, feeding of a hypercholesterolemic diet supplemented with TAU reduced the levels of serum total cholesterol and triglycerides and of liver total lipids, total cholesterol and total triglycerides, and increased the fecal excretion of bile and sterol (Gandhi et al. 1992). Under similar dietary conditions, TAU was also effective in lowering the plasma LDL-cholesterol and hepatic free fatty acid levels (Park and Lee 1998) and in increasing the plasma HDL-cholesterol (Choi et al. 2006; Murakami et al. 2010). The results of work in rats (Murakami et al. 1999, 2010; Yokogoshi et al. 1999) and hamsters (Murakami et al. 2002) have suggested that TAU lowers cholesterol levels by raising the activity as well as the mRNA

expression of hepatic cholesterol 7α-hydroxylase (CYP7A1), the rate-controlling enzyme for the catabolic conversion of hepatic cholesterol to bile acids for subsequent elimination in the feces. Additional hypolipidemic mechanisms attributed to TAU have been interference with the intestinal absorption of cholesterol (Ogawa 1996), a decreasing effect on acyl CoA:cholesterol acyltransferase activity, up-regulation of hepatic LDL receptors and ensuing accelerated LDL turnover (Militante and Lombardini 2004), and a lowering of serum leptin levels (Kim et al. 2012). From *in vitro* studies with HepG2 cells, it appears that TAU interferes with triglycerides synthesis by preventing the incorporation of fatty acid into the glycerol molecule and with VLDL synthesis by reducing the availability of apoB, the major protein component (Yanagita et al. 2008). There is also a suggestion that TAU reduces the hepatic triglyceride content by inhibiting the enzymes diacylglycerol acyl-CoA synthetase and diacylglycerol acyltransferase (Mochizuki et al. 1998; Saleh 2012). Additionally, TAU could be lowering triglycerides by enhancing their peripheral clearance and the activity of the enzyme lipoprotein lipase in the plasma and liver (Nandhini et al. 2002). In the case of MET, this compound may be lowering cholesterol levels by increasing the HDL-cholesterol and the HDL/LDL ratio (Anurag and Anuradha 2002).

Evidence linking hyperglycemia and oxidative stress in either type 1 or type 2 diabetes is abundant. When present in high concentrations glucose can directly contribute to oxidative stress by undergoing autoxidation in the mitochondria to generate ROS or by reacting with proteins in a nonenzymatic manner to form Amadori products followed by AGEs (Schultz Johansen et al. 2005). Additional sources of ROS are the result of the activity of enzymatic pathways such as the polyol pathway or including NAD(P)H oxidase, an established mediator of oxidative stress (Tan et al. 2007). Oxidative stress in type 2 diabetes is of concern because it can contribute to cell injury, to altered cellular physiology and function, and to diabetic complications. Conversely, amelioration of oxidative stress can prevent or attenuate complications of diabetes including nephropathy (King and Loeken 2004). In the kidney of type 2 diabetics glomerular epithelial cells, mesangial cells and proximal tubular epithelial cells may be particularly susceptible to the deleterious consequences of hyperglycemia-induced oxidative stress on account of the increased uptake of circulating glucose in both the postabsorptive and postprandial states and of an increase in glucose reabsorption from the glomerular filtrate in excess of the amounts seen in normal individuals (Meyer et al. 1998; Mitrakou 2011).

In the present study the development of oxidative stress was established based on the levels of MDA, serving as an index of LPO, and of changes in the values of the GSH/GSSG ratio. While all the treatment agents were able to abolish the moderate increase in MDA levels seen in the kidney and plasma of diabetic rats, potencies differences were noted in their ability to attenuate the decreases in kidney and plasma GSH/GSSG ratio of diabetic rats, which was highest with TAU, intermediate with INS, and lowest with MET. A combined treatment with MET plus TAU resulted in an insignificant decrease in the attenuating effect seen with TAU alone. TAU could also be lowering MDA formation through a negative effect on the renal free fatty content, thus making less fatty acid available for lipid peroxidation. In this

connection, it has been reported that TAU can enhance mitochondrial fatty acid oxidation in rats fed a high cholesterol diet (Fukuda et al. 2011) and peroxisomal fatty acid β-oxidation along with a decrease in fatty acid synthase activity in the liver of type 2 diabetic/obese mice (Mikami et al. 2012). These effects may take precedent over the increased lipolysis observed in the adipose tissue of rats treated with TAU (Piña-Zentella et al. 2012). In contrast, MET is found to exert an inhibitory effect on adrenergically-stimulated lipolytic response in adipocytes (Zhang et al. 2009).

In this laboratory, the highly protective effect of TAU on the redox status was previously found to be about equal to that of the GSH biological precursor N-acetylcysteine in the liver of rats receiving a hepatotoxic dose of acetaminophen. More importantly, the increases in GSH and decrease in GSSG levels showed a close correlation with the increases in activity of glutathione reductase and glutathione synthetase (Acharya and Lau-Cam 2010). Preservation of the stores of GSH and scavenging of free radicals has been invoked as a mechanism of protection of TAU against cytotoxicity (Taziki et al. 2013), but this effect is probably exerted indirectly through prevention of the decrease in activity of intracellular antioxidant enzymes (Acharya and Lau-Cam 2010; Pushpakiran et al. 2004) since TAU is a poor radical-trapping agent (Aruoma et al. 1988). The antioxidant action of TAU could also be related to its ability to abolish the increase in renal cortex NAD(P)H oxidase activity (Winiarska et al. 2009).

In alloxan diabetic rabbits, the addition of 1 % TAU to the drinking water resulted in a 30 % decrease in serum glucose, decreased serum urea and creatinine, attenuation of the decline in the GSH/GSSG ratio, the abolishment of the accumulation of hydroxyl radical in the serum and renal cortex, and in increases in renal glutathione reductase and catalase activities (Winiarska et al. 2009). TAU could be limiting lipid peroxidation in an indirect manner by increasing the expression of genes involved in hepatic fatty acid oxidation, thus decreasing the amounts of fatty acids reaching the kidney (Fukuda et al. 2011), or by preserving the intracellular stores of GSH through a positive effect on the activities of GSH-related enzymes such as glutathione reductase and glutathione synthetase (Acharya and Lau-Cam 2010). It has also been proposed that TAU may protect against oxidative stress by serving as a free radical scavenger (Taziki et al. 2013), but this effect is most likely exerted indirectly, possibly by sparing the loss in activity of antioxidant enzymes such as catalase, glutathione peroxidase or superoxide dismutase (Acharya and Lau-Cam 2010) since in itself it is a poor antiradical compound (Aruoma et al. 1988).

In addition to its hypoglycemic properties MET has also demonstrated a direct antioxidant action *in vitro* by inhibiting the production of ROS in human endothelial vascular cells and smooth muscle cells in response to high concentrations of glucose, fatty acid and AGEs (Bellin et al. 2006). The reduction of ROS by MET in endothelial cells exposed to free fatty acids have been ascribed to upregulation of the expression of antioxidant thioredoxin through activation of the AMP-activated protein kinase pathway (Hou et al. 2010). In type 2 diabetics, a 3-month treatment with MET led to a decrease in serum concentrations of advanced oxidation protein products and AGEs, and to an increase in the values of markers of antioxidant

reserve (Esteghamali et al. 2013). Further demonstration of the antioxidant actions of MET on type 2 diabetes was obtained in a study with Goto-Kakizaki rats that found a 4 week treatment with MET to protect the brain against increases in MDA levels, decreases in GSH levels and Mn-SOD activity, and increases in glutathione peroxidase and glutathione reductase activities in addition to its intrinsic hypoglycemic effect (Correia et al. 2008). Evidence on the effects of MET on renal activities of antioxidant enzymes in diabetes seems to be conflicting, with one study demonstrating a lack of effect (Erejuwa et al. 2011) and another one reporting an enhancing effect on the renal normal and diabetic values of catalase, glutathione reductase and GSH (Alhaider et al. 2011). Furthermore, an investigation of the role of MET in the kidney of rats with STZ-induced diabetes disclosed a dose-related down-regulation of the expression of four oxidative stress-mediated genes, GSTα, NQO1, CAT, and HO-1, which plays a crucial role in the pathogenesis of diabetic nephropathy, a decrease in ROS production and an increase in GSH levels (Alhaider et al. 2011). *In vitro*, MET was found to scavenge hydroxyl but not superoxide free radicals and not to react with hydrogen peroxide generated from water by gamma radiolysis (Bonnefont-Rousselot et al. 2003).

Several mechanisms have been identified in cell culture and animal models of diabetes that seem to play a role in the development of diabetic nephropathy in type 1 and type 2 diabetes. All of these mechanisms appear have a persistent hyperglycemia as a common underlying factor (Stanton 2011). The development and outcome of diabetic nephropathy may be the result of several mechanisms, at the center of which are the interaction of hyperglycemia-induced metabolic and hemodynamic alterations and a genetic susceptibility, the activation of various vasoactive systems, an increased secretion of inflammatory molecules, and oxidative stress (Dronavalli et al. 2008). Hyperglycemia can contribute to the development of diabetic nephropathy through various mechanisms. For example, through an overexpression of glucose transporters, glucose accumulates in mesangial cells to cause mesangial cell expansion and hypertrophy, extracellular matrix production, basement membrane thickening, tubular atrophy and interstitial fibrosis (Forbes et al. 2008). Furthermore, an excess of glucose can combine with amino acids in glomerular tissue through a nonenzymatic process to form irreversible advanced glycosylation end (AGE) products that can activate several signal transduction cascades and, thus, alter the levels of signaling molecules such as cytokines, hormones and ROS, modify protein function by entering into cross-linking with collagen or other proteins, or interact with the AGE receptor to reduce the concentrations of vasodilating nitric oxide (Dronavalli et al. 2008; Tan et al. 2007). An additional mechanism may involve the activation of mesangial cell protein kinase C (PKC), known to activate TGF-β1, a promoter of extracellular matrix production in mesangial cells (Riser et al. 1999) and mitogen-activated protein kinase, a protein downstream of PKC, known to enhance the production of eicosanoids associated with glomerular hyperfiltration (Haneda et al. 1995). Also, a moderate hyperglycemia without glycosuria can enhance plasma renin activity and mean arterial pressure in those with uncomplicated type 1 diabetes. As a result, there will be hyperfiltration and an increase in glomerular pressure which, presumably, can damage glomerular cells and cause glomerulosclerosis (Stanton 2011).

In diabetes renal glomerular mesangial and tubular epithelial cells are stimulated by hyperglycemia to produce ROS. Both NADP oxidase and mitochondrial electron transport play major roles in hyperglycemia-induced ROS production. ROS not only contribute to the development of diabetic glomerular lesions by upregulating TGF-β, the key mediator of extracellular matrix production and accumulation in the glomerulus, but also can act as intracellular messengers capable of activating signal transduction cascades and transcription factors for the upregulation of profibrotic genes like TGF-β and proteins involved in glomerular mesangial expansion and tubulointerstitial fibrosis (Ha et al. 2008; Lee et al. 2003).

Serum markers of glomerular filtration rate and the extent of albumin loss into the urine identify and provide an estimate of the progress of the renal impairment in different segments of the diabetic population. At the start, there is an increase in kidney size, damage and normal or increased glomerular filtration rate. With time, small amounts of blood albumin escape into the urine to cause microalbuminuria. As the disease progresses the rate of albumin excretion worsens and becomes macroalbuminuria or proteinuria. With an increase in the severity of the albuminuria there is a progressive decline in the glomerular filtering capacity and various metabolic waste products are retained in the circulation (Dabla 2010).

In the present study, STZ-diabetic rats exhibited a much higher 24 h urine output than normal rats probably in response to the high blood glucose and accompanying increase in plasma osmolarity. This effect was accompanied by a heavy proteinuria and a significant reduction in the plasma total protein level. The significant leakage of systemic proteins into the urine seen in STZ-treated animals has been ascribed in part to a direct toxic effect by STZ and mostly to renal morphologic and ultrastructural abnormalities caused by a prolonged hyperglycemia (Hall-Craggs et al. 1982; Palm et al. 2004). INS, MET, MET-TAU and, to slightly lesser extent, TAU were able to reduce the daily urinary output to a significant extent, with the effect of INS being about two times higher than the other treatments. The same order of potency was noted in terms of the preservation of proteins in the circulation and reduction of proteins in the urine. A critical role for hyperglycemia in the onset and progression of diabetic nephropathy was suggested by the ability of INS to normalize the urinary protein output and of MET to reduce protein leakage into the urine. At the same time, these findings confirm the importance of an adequate glycemic control as a protection against diabetic nephropathy (Iglesias and Díez 2008). Although TAU attenuated diabetic proteinuria, its effect was weaker than that of MET and probably less dependent on a hypoglycemic effect. The renoprotective effects of this amino acid may be related at least in part to its antioxidant properties (Wang et al. 2008).

Further evidence of renal dysfunction in diabetic rats was suggested by the profound increases in the plasma creatinine and urea nitrogen levels. Again, all the treatment agents were highly protective but their potencies varied according to the particular agent. Lower retentions of creatinine and urea nitrogen were observed in the presence of INS and TAU than of MET or TAU-MET.

In diabetic patients an increase in serum glucose is usually accompanied by an increase in serum osmolality and in Na^+ and K^+ levels (Rao 1992; Shahid et al. 2005) although a decrease in serum Na^+ has also been reported (Al-Rubeaan et al. 2011; Wang et al. 2013). Moreover, in type 2 diabetic patients the severity of the

hyperkalemia has been shown to directly correlate with the degree of hyperglycemia (Al-ajlan 2010). In this work, diabetes was found to elevate the plasma Na^+ and K^+ to concentrations significantly higher than those of normal rats. TAU and MET were found to effectively counteract these changes, with TAU appearing equipotent with MET on the plasma Na^+ and more potent than MET on the plasma K^+. Although a combined treatment with MET plus TAU led to an enhancement in potency over the individual treatments, the difference were not significant. An identical trend in the changes in plasma Na^+ and K^+ and in the protective actions of MET was reported by Baxi et al. (2010) using alloxan-treated albino rats. The beneficial effects of both MET and TAU on the renal function of diabetic rats may reflect their ability to prevent histological changes associated with glomerular abnormalities (Alhaider et al. 2011; Pandya et al. 2013; Winiarska et al. 2009).

TGF-β1 is a multifunctional protein that regulates inflammation and connective tissue synthesis under conditions of high ROS and glucose concentrations. More specifically, TGF-β1 can induce the accumulation of fibrotic tissue in the extracellular matrix of the glomeruli in response to high glucose, with ROS appearing to amplify TGF-β1 signaling through a key glycoprotein known as plasminogen activator inhibitor-1, minimally expressed in normal human kidney cells but overexpressed in the diabetic kidney (Lee et al. 2005). Work with mice that are transgenic for TGF-β1 have disclosed that increased levels of circulating TGF-β1 can induce progressive renal disease characterized by mesangial expansion, accumulation of glomerular immune deposits and matrix proteins, interstitial fibrosis, proteinuria, progressive azotemia and uremic death (Kopp et al. 1996). The measurement of plasma TGF-β1 in the present study indicated a massive increase in untreated diabetic rats, a minimal increase in INS-treated diabetic, and a moderate increase in diabetic rats receiving either MET or TAU. A combined treatment with MET plus TAU led to a small, yet significant, gain in potency relative to the individual treatments. This result contrasts markedly with the lack of effect demonstrated by MET on the increased serum levels of TGF-β1 seen in normoalbuminuric and normotensive patients with type 2 diabetes (Yener et al. 2008). On the other hand, MET was reported to be effective in reducing the elevations in active TGF-β1 in type 2 diabetics when administered along with INS, galargine or a DPP-4 inhibitor but not with a sulfonylurea (Pscherer et al. 2013). TAU has also been found to significantly reduce the mRNA expression of TGF-β1 in rats with experimental nonalcoholic steatohepatitis (Chen et al. 2006) and in rats with carbon tetrachloride-induced hepatic fibrosis (Miyazaki et al. 2005). The present results lend support to TAU as a protectant against diabetes-induced renopathy.

5 Conclusion

The present results verify that MET and TAU can effectively protect against diabetes-induced alterations in glucose and lipid metabolism, renal oxidative state, loss of renal function, and increases in TGF-β1 levels. Except for the greater effect of MET over TAU in terms of glucose-related variables, the two compounds

protected the kidney against remaining changes in a rather similar manner and extent. A combined treatment with MET plus TAU was more protective against dyslipidemia and changes in redox status than MEt alone. Other than changes in glucose-related parameters, MET, TAU or their combination generally provided protective effects against diabetes that were of about the same or greater magnitude than those of INS. Hence, further evaluation of TAU as an adjunct of MET in the prevention of diabetes-related complications is clearly warranted.

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Evaluation of the Actions of Metformin and Taurine, Singly and in Combination, on Metabolic and Oxidative Alterations Caused by Diabetes in Rat Erythrocytes and Plasma

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Abbreviations

CAT	Catalase
GPx	Glutathione peroxidase
GSH	Reduced glutathione
GSSG	Glutathione disulfide
HbA_{1c}	Glycated hemoglobin
INS	Insulin
MDA	Malondialdehyde
MET	Metformin
RBCs	Erythrocytes
SOD	Superoxide dismutase
STZ	Streptozotocin
TAU	Taurine

1 Introduction

Oxidative stress is recognized as one the major contributing factors to the pathogenesis of type 1 and type 2 diabetes. In patients with diabetes there is an excessive formation of reactive oxygen species (ROS) and reactive nitrogen species (NOS), a decrease in antioxidant defenses, and changes in the rates of stress-sensitive metabolic, proinflammatory and proapoptotic pathways. Under these conditions,

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organelles, biomolecules and membranes become damaged, lipid peroxidation (LPO) increases, cellular homeostasis is disturbed, insulin secretion and biosynthesis decreases, and insulin resistance develops (Evans et al. 2003; Giacco and Browlee 2010; Jakus 2000; Maritim et al. 2003). Moreover, a correlation has been established between hyperglycemia, oxidative stress and the development of diabetic complications in type 1 and type 2 diabetes, and of insulin resistance and impaired insulin secretion in type 2 diabetes (Brownlee 2005; Evans et al. 2003; Giacco and Browlee 2010).

Hyperglycemia is regarded as a trigger for oxidative stress (Choi et al. 2008) and as the common linkage to a diverse group of mechanisms that have been implicated in the development of diabetic complications, including the production of superoxide radicals by mitochondria, of proinflammatory cytokines by protein kinase C-activated pathways, and of advanced glycation products (Brownlee 2005; Giacco and Browlee 2010). This concept is supported by the results of studies in diabetic patients indicating that oxidative stress is significantly reduced upon a satisfactory glycemic control (DDCT 1993; Grieco et al. 2013; Motawi et al. 2013). However, studies with diabetic patients and animal models of diabetes have also suggested that intensive hypoglycemic therapy or the achievement of a euglycemic state can at best only attenuate and not to completely reverse the changes in biochemical indices of oxidative stress or improve the redox status (Erejuwa 2012).

Based on the recognition that oxidative stress is an independent entity in diabetes that exacerbates the development and progression of diabetic complications, a wide range of compounds with intrinsic antioxidant properties have been evaluated for the ability to prevent or reduce diabetes-related oxidative stress when used alone or in combination with insulin or conventional oral hypoglycemic agents (Maritim et al. 2003). One of the compounds that has been extensively studied for this purpose is taurine (TAU), a β-amino acid known to attenuate oxidative stress from different sources by inhibiting plasma and tissue lipid peroxidation, maintaining the intracellular glutathione redox status, preserving the activity of antioxidant enzymes (Acharya and Lau-Cam 2010), diminishing hyperlipidemia (Goodman and Shihabi 1990), and preventing the formation of advanced glycation end products (AGEs) (Nandhini et al. 2004; Trachtman et al. 1995). More importantly, by forming a conjugate with mitochondrial transfer RNA, exogenous TAU has been shown to induce the translation and expression of mitochondrial-encoded protein components of the electron transport chain, which may have become limiting upon a shortage of endogenous TAU, needed to restore ATP synthesis at the expense of superoxide anion formation (Schaffer et al. 2009). In addition, work in animal models of diabetes have uncovered additional beneficial actions for this sulfur-containing compound such as a hypoglycemic effect (Winiarska et al. 2009), enhancement of peripheral insulin sensitivity and glucose tolerance (Kulakowski and Maturo 1984; Lampson et al. 1983), and a stimulatory action on insulin secretion (Tenner et al. 2003). Studies in overweight men with a positive history of type 2 diabetes (Brøns et al. 2004) or in Otsuka Long-Evans Tokushima Fatty rat, a model of spontaneous diabetes (Nakaya et al. 2000) have shown that a daily supplementation with TAU can improve insulin sensitivity. The

same effect has been observed when TAU was administered in combination with an antioxidant like N-acetylcysteine (Odetti et al. 2003; Patriarca et al. 2006). In addition, a co-treatment with TAU and a ginseng preparation was found to exert a greater effect on hyperglycemia, glycated hemoglobin formation, insulin hyposecretion, dyslipidemia and increases in the serum values of enzyme markers of cardiac damage and of endothelin-1 associated with STZ-induced diabetes in rats than either treatment alone (Saleh 2012).

While the scientific literature on the antioxidant properties of TAU is rather extensive, that on the effect of adding TAU to a treatment with an oral hypoglycemic to our knowledge remains to be investigated. To clarify this situation, the present study was undertaken in rats made diabetic with streptozotocin (STZ) to compare TAU with metformin (MET), a current drug of choice for the management of type 2 diabetes and demonstrating antioxidant properties, for their effects on diabetes-induced oxidative stress. In addition, the merits of a combined treatment with MET and TAU against those of MET alone were also examined. To this end, observable biomarkers of oxidative stress, including the enzymatic activities of catalase, glutathione peroxidase and superoxide dismutase, the changes in reduced glutathione and glutathione disulfidelevels, and the formation of malondialdehyde, serving as an index of lipid peroxidation, were measured in the plasma and erythrocytes of diabetic rats treated daily with MET, TAU or MET-TAU for 6 weeks after the induction of diabetes and the results were correlated with the corresponding plasma glucose, plasma insulin and blood glycated hemoglobin levels.

2 Methods

2.1 Animals and Treatments

Groups of six male Sprague-Dawley rats, 225–250 g, were used in the experiments. Diabetes was induced on day 1 with an intraperitoneal, 60 mg/kg, dose of STZ, dissolved in 10 mM citrate buffer, pH 4.5. The diabetic rats were treated on a daily basis with a single, 2.4 mM/kg, oral dose of MET, TAU or MET plus TAU (dissolved in physiological saline) or with a single 4 U/kg, subcutaneous dose of INS (70 % NPH insulin suspension, Humulin N®). Control rats received only 10 mM citrate buffer pH 4.5 or only STZ. All the treatments with MET, TAU or their combination were started on day 15 and continued until day 56. The progress of the diabetes was monitored by weekly measuring the concentration of glucose in a drop of tail vein glucose using a glucometer (TRUEtrack™ and test strips, Nipro Diagnostics, Fort Lauderdale, FL). The study was approved by the Institutional Animal Care and Use Committee of St. John's University, Jamaica, NY, and the animals were cared in accordance with guidelines established by the United States Department of Agriculture.

2.2 Samples and Assays

Blood samples were collected into heparinized tubes on day 57 following decapitation. One portion was used to measure the concentration of glycated hemoglobin (HbA_{1c}). The other portion was used to obtain plasma and RBCs. The plasma was analyzed for glucose, hemoglobin, HbA_{1c} and indices of oxidative stress; and the RBCs, processed as described by Sharma and Premachandra (1991), were analyzed for hemoglobin content, membrane cholesterol to phospholipids ratio, and indices of oxidative stress.

The plasma glucose was measured with a commercially available colorimetric assay kit (procedure No. 510, Sigma-Aldrich, St. Louis, MO). An ELISA assay kit (Insulin ELISA, Calbiotech Inc., Spring Valley, CA) was used to measure the plasma INS level. Blood HbA_{1c} was measured by means of a commercial colorimetric assay kit (Glycohemoglogin Direct Test, Stanbio Laboratory, Boerne, TX). The extent of LPO in the plasma and RBC was assessed on the basis of the MDA levels, measured as thiobarbituric acid reactive substances (TBARS), by the colorimetric end-point method of Buege and Aust (1978). The contents of GSH and GSSG in the plasma and RBC were measured using the fluorometric method of Hissin and Hilf (1976), which is based on the reaction of *ortho*-phthalaldehyde with GSH at pH 8 and with GSSG at pH 12.0, using the method of Güntherberg and Rost (1966) to remove any interfering preformed GSH prior to the assay of GSSG. The concentration of free hemoglobin (Hb) in the plasma and RBC was measured with a commercial spectrophotometric assay kit (Procedure No. 525-A, Sigma-Aldrich, St. Louis, MO), based on the oxidation of hemoglobin to methemoglobin with Drabkin's reagent. The plasma and RBC activities of CAT, GPx and Cu,Zn-SOD were measured according to the methods of Aebi (1984), Günzler and Flohé (1985) and Misra and Fridovich (1972), respectively. The plasma concentration of cholesterol was determined with a colorimetric enzymatic assay kit (Cholesterol LiquiColor®, Procedure No. 1010, Boerne, TX). RBCs for the assay of membrane cholesterol and phospholipids were first made into a suspension in Tris-HCl buffer pH 7.4 and then extracted with chloroform as described by Folch et al. (1957). Evaporation of the chloroform solution yielded residues suitable for the assay of membrane cholesterol, as described for the plasma, and of membrane phospholipids, by the colorimetric method of Stewart (1979).

2.3 Statistical Analysis of Data

The experimental results are reported as mean ± standard error of the mean (SEM) for $n = 6$ rats. The results were analyzed for statistical significance using unpaired Student's t-test followed by one-way analysis of variance (ANOVA) and Tukey's post hoc test. Intergroup differences were considered to be statistically significant when $p \leq 0.05$.

3 Results

3.1 *The Effect of MET, TAU and MET-TAU on Plasma Glucose*

At the end of 56 days rats treated with STZ showed a dramatic increase in their plasma glucose level (by ~4.1-fold, $p < 0.001$) over the control value (Table 1). A daily, 6-week treatment with MET reduced this elevation by more than one-half (only ~1.9-fold increase, $p < 0.001$ vs. diabetes). An identical treatment with TAU also resulted in a significant attenuation of the hyperglycemic response caused by diabetes but to a much lesser extent (~3.2-fold increase) than MET. However, when TAU was administered alongside MET, the reduction in hyperglycemic response was about equal to MET alone (1.9-fold). Daily doses of INS, on the other hand, reduced the diabetic plasma glucose to a value that was only 23 % above the control value ($p < 0.05$) (Table 1).

3.2 *The Effect of MET, TAU and MET-TAU on Plasma INS*

In diabetic rats, the plasma INS level at the end of 56 days was much lower (by 75 %, $p < 0.001$) than the control value (Table 1). Treating the diabetic rats with MET raised the plasma INS to a significant extent (by >3-fold, $p < 0.001$) compared to the value of untreated diabetic rats. TAU was also very effective in raising the diabetic plasma INS but not as much as MET (~2-fold higher, $p < 0.001$ vs. diabetes). The plasma INS level of diabetic rats receiving a daily dose of INS was significantly higher than the baseline value (by 21 %, $p < 0.05$) at the end of 56 days (Table 1).

Table 1 The effect of INS, MET, TAU and TAU-MET on the plasma glucose and INS and on the blood HbA_{1c} of diabetic rats[a]

Treatment group	Glucose, mg/dL	INS, μIU/mL	HbA_{1c}, %
Control	$103.59 \pm 5.06^{+++}$	$44.08 \pm 2.46^{+++}$	$6.89 \pm 0.11^{+++}$
DM	$428.08 \pm 21.77^{***}$	$10.58 \pm 1.33^{***}$	$21.14 \pm 1.19^{***}$
DM-INS	$127.15 \pm 10.71^{*,+++}$	$53.46 \pm 6.97^{*,+++}$	$7.46 \pm 0.41^{***,+++}$
DM-MET	$196.41 \pm 5.00^{***,+++}$	$32.50 \pm 2.35^{**,+++}$	$9.77 \pm 0.41^{+++}$
DM-TAU	$333.27 \pm 6.60^{***,+}$	$20.94 \pm 1.83^{***,+++}$	$10.96 \pm 0.96^{***,+++}$
DM-TAU-MET	$190.43 \pm 2.22^{***,+++}$	$36.02 \pm 1.87^{*,+++}$	$7.65 \pm 1.04^{+++}$

Values are reported as the mean ± SEM for n = 6

[a]Data were analyzed using unpaired Student's t-test followed by ANOVA and Tukey's *post hoc* test. Statistical comparisons were vs. Control at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$; vs. STZ at $^{+}p < 0.05$ and $^{+++}p < 0.001$

3.3 The Effect of MET, TAU and MET-TAU on Blood HbA_{1c}

Diabetes elevated the blood HbA_{1c} concentration drastically (>200 %, $p<0.001$) in comparison to the value of control rats (Table 1). A treatment with either MET or INS lowered the diabetic HbA_{1c} to a value that was not significantly different from that of naive rats (increases of only 13 % and 8 %, respectively). By itself, TAU was also very protective but to a much lesser extent than MET or INS (59 % increase, $p<0.001$ vs. control). However, when administered alongside MET, it led to a blood HbA_{1c} comparable to that with MET (11 % increase) (Table 1).

3.4 The Effect of MET, TAU and MET-TAU on Plasma and RBC MDA

The occurrence of LPOwas investigated by measuring the plasma levels of MDA as TBARS. As shown in Fig. 1, diabetic rats showed a much higher plasma MDA value (by ~40 %, $p<0.001$) than normal rats. A treatment with MET, TAU, MET-TAU or INS was found to virtually abolish the formation of plasma MDA (only 3–6 % increase).

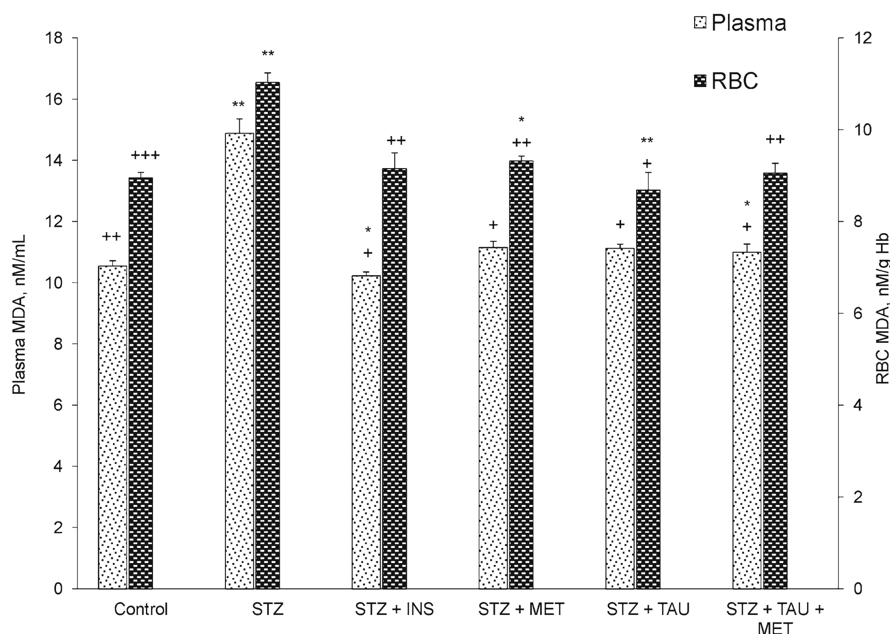

Fig. 1 The effect of a 6 week daily treatment with MET, TAU and MET-TAU, started at 14 days after the induction of diabetes with STZ, on the diabetic plasma and RBC MDA levels on day 56. INS served as a reference treatment. Data are shown as mean ± SEM for n = 6. Differences were significant vs. Control at $^{*}p<0.05$ and $^{**}p<0.01$; and vs. STZ at $^{+}p<0.05$, $^{++}p<0.01$ and $^{+++}p<0.001$.

A good correlation existed between the plasma and RBC levels of MDA. In the RBCs, however, although the increase in diabetic rats was still significant relative to the control value, it was, however, less pronounced (23 % increase, $p<0.05$) (Fig. 1). Again, all the treatment agents, including INS, were able to virtually eliminate the formation of MDA (up by ≤4 %) (Fig. 1).

3.5 The Effect of MET, TAU and MET-TAU on the Plasma and RBC GSH, GSSG and GSH/GSSG Ratios

The influence of MET and INS on the oxidative stress of diabetic rats was determined by measuring the levels of GSH and GSSG and by calculating the corresponding GSH/GSSG ratios. From the results presented in Figs. 2 and 3, it is apparent that diabetes had a significant lowering effect on the plasma GSH and a significant elevating effect on the plasma GSSG, respectively. As a result, the GSH/

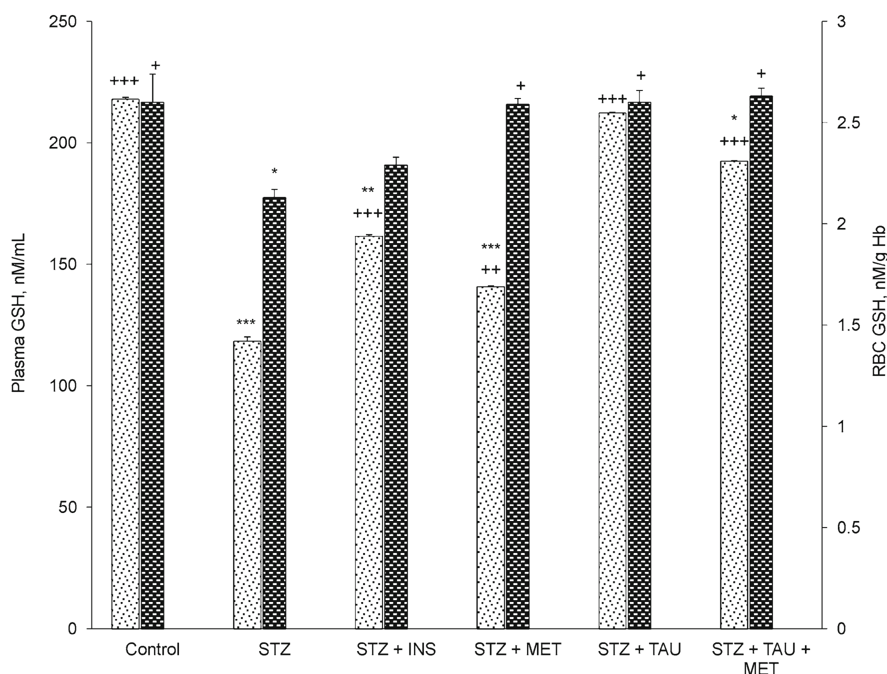

Fig. 2 The effect of a 6 week daily treatment with MET, TAU and MET-TAU, started at 14 days after the induction of diabetes with STZ, on the diabetic plasma and RBC GSH levels on day 56. INS served as a reference treatment. Data are shown as mean ± SEM for n = 6. Differences were significant vs. Control at $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$; and vs. STZ at $^{+}p<0.05$, $^{++}p<0.01$ and $^{+++}p<0.001$.

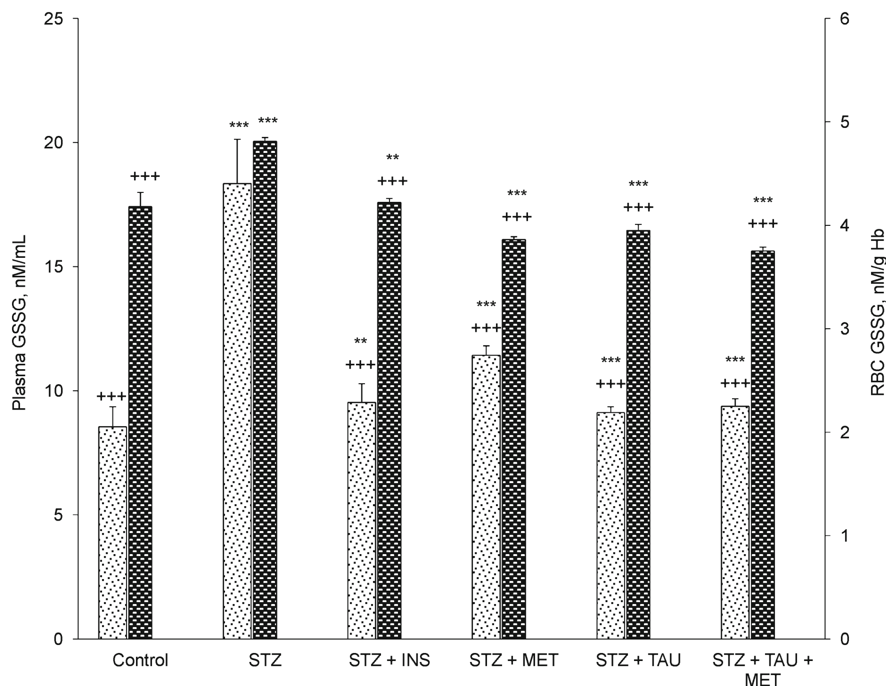

Fig. 3 The effect of a 6 week daily treatment with MET, TAU and MET-TAU, started at 14 days after the induction of diabetes with STZ, on the diabetic plasma and RBC GSSG levels on day 56. INS served as a reference treatment. Data are shown as mean ± SEM for n = 6. Differences were significant vs. control at $^{**}p < 0.01$ and $^{***}p < 0.001$; and vs. STZ at $^{+++}p < 0.001$.

GSSG ratio was significantly decreased (by 75 %, $p<0.001$) compared to the control value (Fig. 4). In contrast, TAU, and to a lesser extent MET, were able to effectively counteract the change in plasma GSH/GSSG ratio caused by diabetes (9 % and ~50 % decreases, respectively, vs. control). Moreover, a combined treatment with MET plus TAU was much more effective than MET alone (only 19 % decrease, $p<0.05$) and also than INS (33 % decrease, $p < 0.01$ vs. diabetes in stabilizing the GSH/GSSG ratio) (Fig. 4).

In comparison to its effects on the plasma GSH and GSSG contents, diabetes affected the corresponding RBC values to a more limited extent (Figs. 2 and 3). Relative to the control values, the GSH was lowered by 18 % ($p < 0.05$) (Fig. 2) the GSSG was increased by 15 % ($p < 0.05$) (Fig. 3), and the corresponding GSH/GSSG ratio was decreased by 23 % ($p < 0.05$) (Fig. 4). In contrast, all the treatment agents and approaches were able to reverse this trend, with MET and TAU bringing the ratio to a value ~7 % over control when administered separately and 13 % over control when administered together. In this regard, INS was also protective but was not able to reverse the decline in the GSH/GSG ratio to the same extent as MET or TAU (13 % decrease) (Fig. 4).

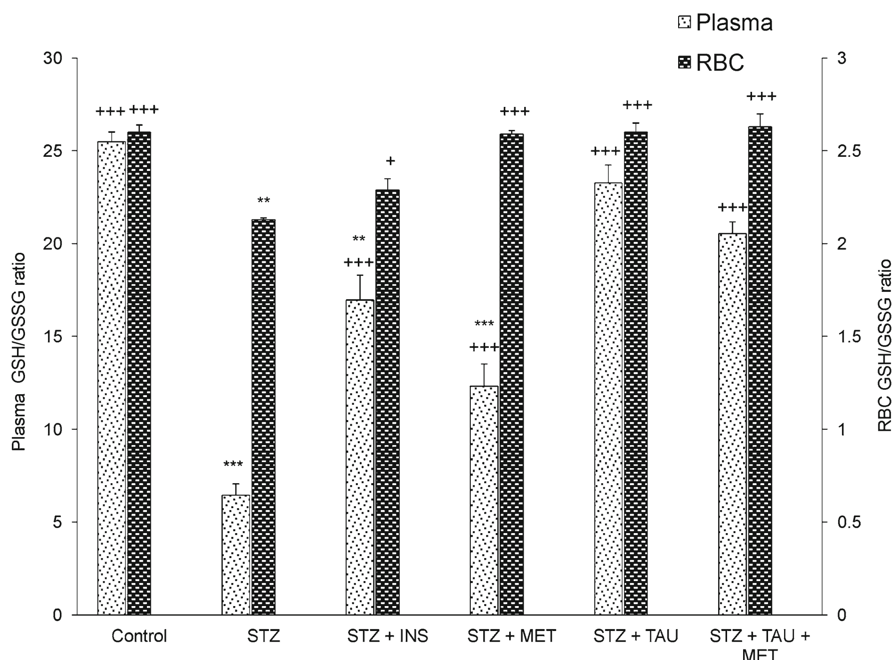

Fig. 4 The effect of a 6 week daily treatment with MET, TAU and MET-TAU, started at 14 days after the induction of diabetes with STZ, on the diabetic plasma and RBC GSH/GSSG ratios on day 56. INS served as a reference treatment. Data are shown as mean ± SEM for n = 6. Differences were significant vs. control at $^{**}p<0.01$ and $^{***}p<0.001$; and vs. STZ at $^{+}p<0.05$ and $^{+++}p<0.001$.

3.6 The Effect of MET, TAU and MET-TAU on the Activities of Plasma and RBC Antioxidant Enzymes

Figures 5, 6, and 7 show the changes in plasma antioxidant enzymes under the influence of diabetes. After 56 days, the plasma activities of CAT, GPx and SOD were significantly decreased relative to the corresponding control values (by 58 %, 63 % and 66 %, respectively, all at $p<0.001$ vs. controls). All the treatment compounds were found to attenuate these decreases, with the potency differences between MET and TAU varying according to the particular enzyme. TAU was more potent than MET in raising the diabetic values of CAT (by 84 % vs. 48 %) (Fig. 5) and SOD (by 110 % vs. 75 %) (Fig. 6); but MET was more potent than TAU in raising the values of GPx (by 135 % vs. 79 %) (Fig. 7). Treating the diabetic rats with MET plus TAU led to a dramatic increase ($p<0.001$) in the protective effects derived from TAU on the CAT (up by 102 %) and SOD (up by 115 %) activities and from MET on the GPx activity (up by 144 %) of the diabetic rats. In the presence of INS, the diabetic plasma activities of the antioxidant enzymes increased in a variable manner, being closer to the effects of TAU on CAT (98 % increase) and GPx (109 % increase), and better than TAU (165 % increase) in the case of SOD (Figs. 5, 6, and 7, respectively).

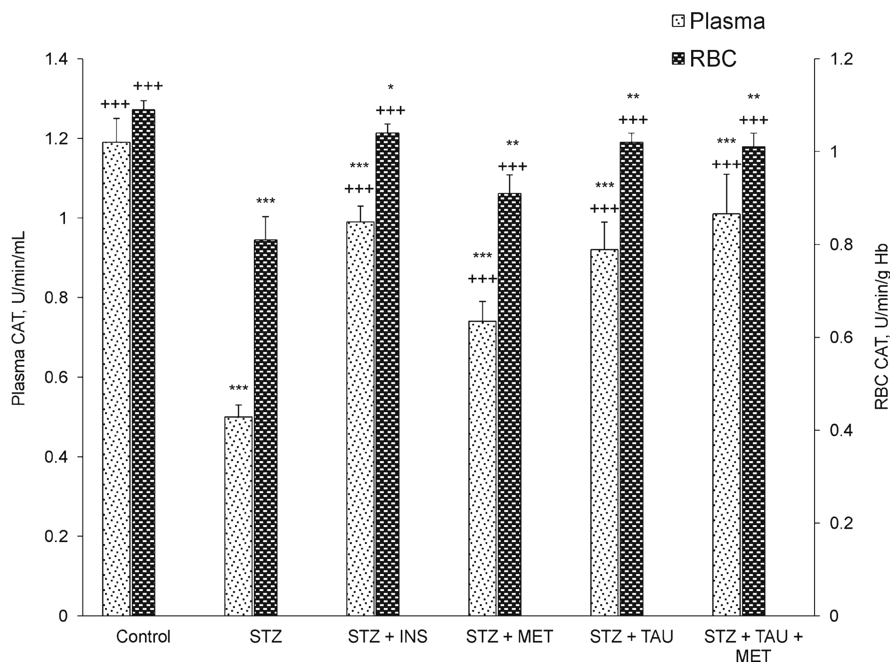

Fig. 5 The effect of a 6 week daily treatment with MET, TAU and MET-TAU, started at 14 days after the induction of diabetes with STZ, on the diabetic plasma and RBC CAT activities on day 56. INS served as a reference treatment. Data are shown as mean ± SEM for n = 6. Differences were significant vs. control at $^{**}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$; and vs. STZ at $^{+++}p<0.001$.

In parallel with the decreases in the activities of CAT, GPx and SOD seen in the plasma of diabetic rats, those in the RBCs were also lower than control values although to a more limited extent (decreases of 26 %, 35 % and 47 %, respectively, $p \leq 0.01$ vs. control values) (Figs. 5, 6, and 7, respectively). At variance with the findings for the plasma, TAU was found to exert a greater elevating effect than MET on the RBC diabetic activities of CAT (by 26 %, $p<0.05$ vs. 12 % increases) (Fig. 5), SOD (70 % vs. 53 % increases, both at $p<0.001$) (Fig. 6) and GPx (27 %, $p<0.01$ vs. 18 %, $p<0.05$ increases) (Fig. 7) and relative to the diabetic activities. A treatment with MET plus TAU provided a protective action on all three enzymes that was equivalent to that by TAU alone, an effect that was also encountered upon a treatment with INS (Fig. 5, 6, and 7).

3.7 The Effect of MET, TAU and MET-TAU on Plasma and RBC Hb

The occurrence of diabetes was accompanied with a significant leakage of Hb from the RBCs into the circulation. As a result, an inverse relationship could be

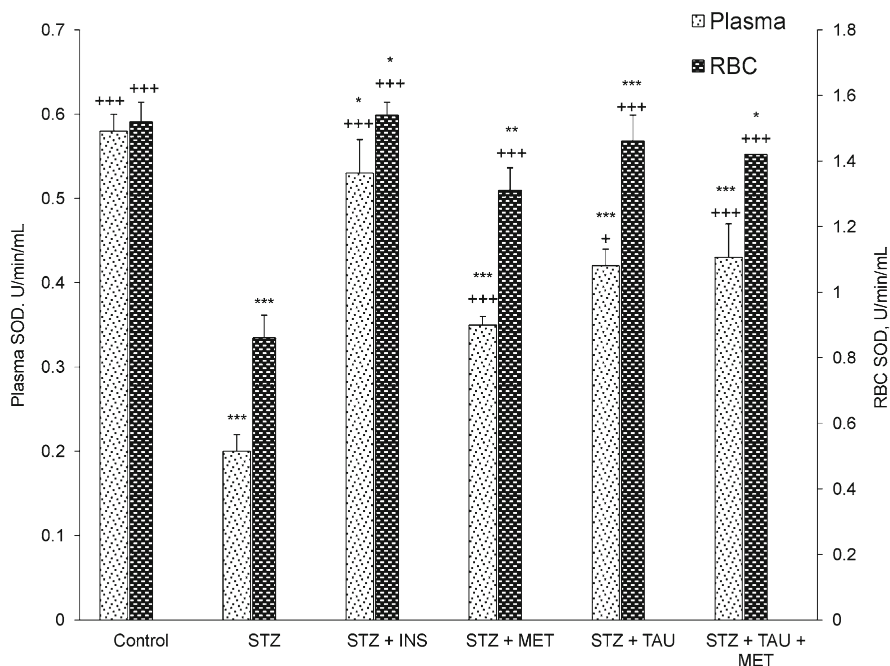

Fig. 6 The effect of a 6 week daily treatment with MET, TAU and MET-TAU, started at 14 days after the induction of diabetes with STZ, on the plasma and RBC SOD activities on day 56. INS served as a reference treatment. Data are shown as mean ± SEM for n = 6. Differences were significant vs. control at $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$; and vs. STZ at $^{+}p<0.05$ and $^{+++}p<0.001$.

established between the concentrations of Hb in the plasma and RBCs from diabetic rats, being significantly increased in the plasma (by 100 %) (Fig. 8) and decreased in the RBC (by 42 %) (Fig. 8) in comparison to the respective control values. Treating the diabetic rats with MET, TAU or MET-TAU led to minimal changes in the baseline plasma and RBC Hb levels (≤6 % in the plasma, ≤8 % in the RBC). A treatment with INS resulted in an effect similar to that by MET or TAU (increases of only 3 % increase in the plasma and of 7 % increase in the RBC) (Fig. 8).

3.8 The Effect of MET, TAU and MET-TAU on the RBC Membrane Cholesterol to Phospholipids Ratio

The RBC membrane of diabetic rats showed a significantly increase (by 125 %, $p<0.001$) in its cholesterol content over membranes from naive rats (Fig. 9). A treatment with MET or TAU resulted in a significant lowering of the diabetic membrane cholesterol (by 20 % and 21 %, respectively, $p<0.05$) The administration of MEt along with TAU led to an enhancement of the effect seen with the individual

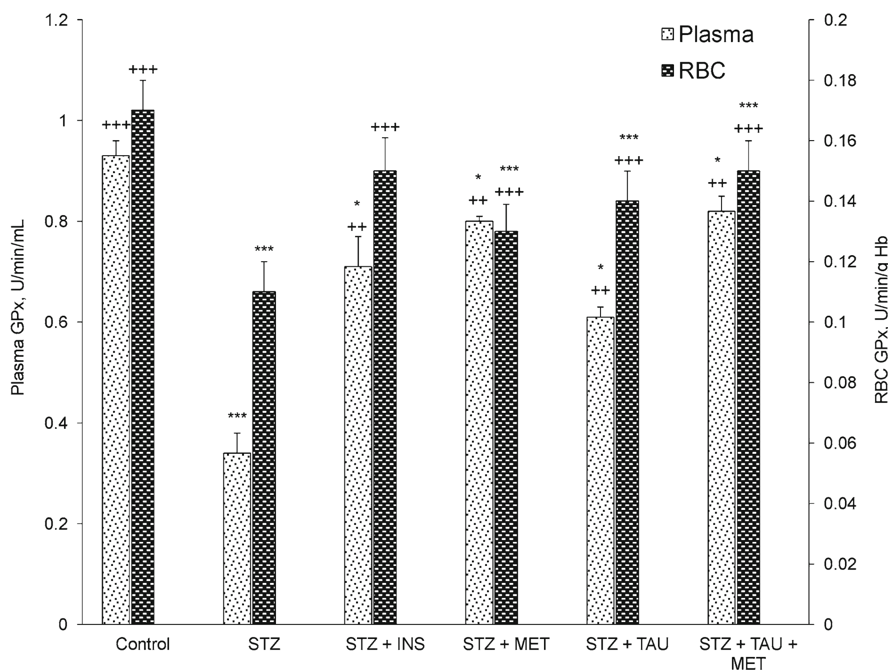

Fig. 7 The effect of a 6 week daily treatment with MET, TAU and MET-TAU, started at 14 days after the induction of diabetes with STZ, on the diabetic plasma and RBC GPx activities on day 56. INS served as a reference treatment. Data are shown as mean ± SEM for n = 6. Differences were significant vs. control at $^{*}p<0.05$ and $^{***}p<0.001$; and vs. STZ at $^{++}p<0.01$ and $^{+++}p<0.001$.

treatments (31 % less, $p<0.01$ vs. diabetes); and a treatment with INS provided a protection about equal to that derived from either MET or TAU (21 % less, $p<0.05$ vs. diabetes) (Fig. 9).

Diabetes also affected the diabetic RBC membrane phospholipids content, which, although mild, was still significantly higher than the control value (22 % increase, $p<0.05$) (Fig. 9). A treatment with TAU was insignificantly better than one with MET (increases of 9 % and 15 %, respectively). On the other hand, a treatment with MET plus TAU was better than the individual treatments (only 4 % increase). INS demonstrated an effect about equal to that of MET (14 % increase) (Fig. 9).

Due to its effects on the RBC membrane cholesterol and phospholipid contents, diabetes raised the cholesterol/phospholipids ratio considerably above the control value (by 85 %, $p<0.001$) (Fig. 10). Treatments with MET (only 58 % increase), TAU (63 % increase), or better with MET plus TAU (49 % increase) were found to reduce the diabetic cholesterol/phospholipids ratio significantly (all at $p<0.001$ vs. diabetes). A treatment with INS exerted a lowering effect similar to that by MET (57 % increase) (Fig. 10).

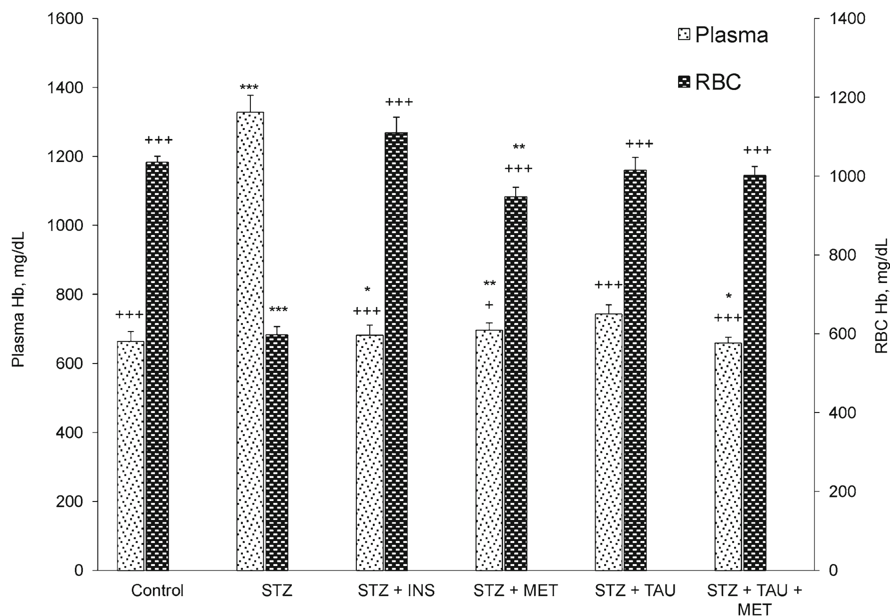

Fig. 8 The effect of a 6 week daily treatment with MET, TAU and MET-TAU, started at 14 days after the induction of diabetes with STZ, on the diabetic plasma and RBC Hb levels on day 56. INS served as a reference treatment. Data are shown as mean ± SEM for n = 6. Differences were significant vs. control at $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$; and vs. STZ at $^{+}p<0.01$ and $^{+++}p<0.001$.

4 Discussion

This study has examined the actions of a daily oral treatment with TAU, MET or TAU-MET (2.4 mM/kg each) for 42 days on the changes in circulating glucose, INS and HbA_{1c} levels, in markers of oxidative stress in the plasma and RBCs, and in the integrity and lipid composition of the RBC membrane after diabetes had been established with STZ. For comparative purposes, a separate group of diabetic rats received a daily subcutaneous dose (4 U/kg) of isophane INS. In this regard, RBCs are very appropriate for this type of study since they contain high concentrations of oxygen and Hb. As a consequence of their high oxygen content, RBCs are able to generate their own free radicals via process termed autoxidation and in which oxyhemoglobin is degraded to release superoxide anion, which subsequently reacts with oxyhemoglobin to also produce H_2O_2 (Winterbourn 1985). Moreover, the prolonged contact of RBCs with high blood glucose levels can increase membrane protein glycation, reduce ATPase activity, lower antioxidant enzymes activity, cause the redistribution of constitutive phospholipids and reduce fluidity (Stanescu et al. 2002). These changes have been found amenable to attenuation by a treatment with INS (Juhan-Vague et al. 1986).

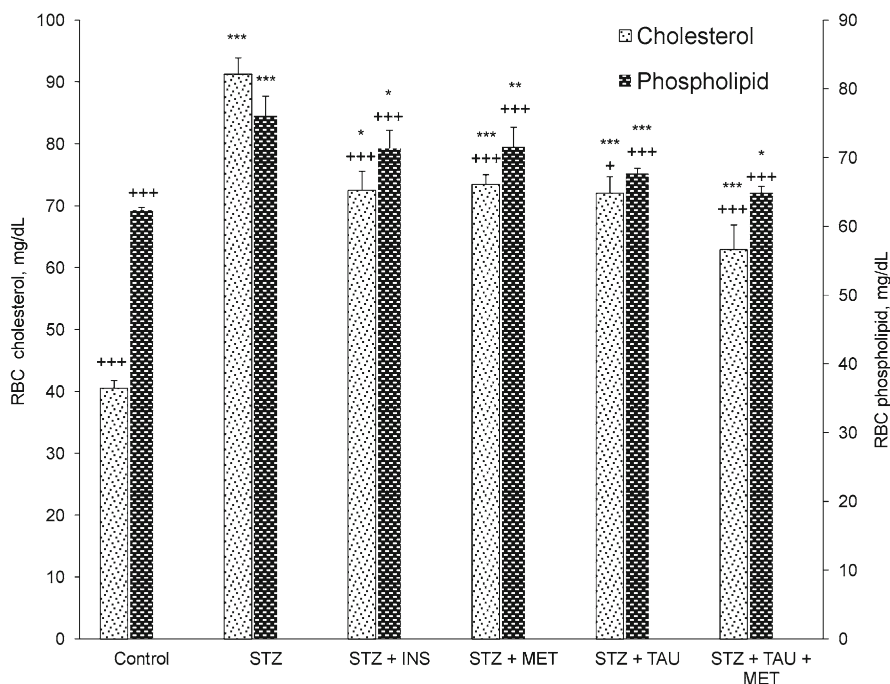

Fig. 9 The effect of a 6 week daily treatment with MET, TAU and MET-TAU, started at 14 days after the induction of diabetes with STZ, on the diabetic RBC membrane cholesterol and phospholipids levels on day 56. INS served as a reference treatment. Data are shown as mean ± SEM for n = 6. Differences were significant vs. control at $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$; and vs. STZ at $^{+}p<0.05$ and $^{+++}p<0.001$.

The effects of TAU on the circulating glucose levels is conflicting, with some laboratories reporting a significant lowering effect (Kaplan et al. 2004; Rashid et al. 2013; Winiarska et al. 2009), a few reporting a slight although insignificant effect (Tenner et al. 2003), and some reporting no effect (Ersöz et al. 1994; Goodman and Shihabi 1990; Obrosova et al. 2001). The present study found TAU to be able to reduce the diabetic glucose level to a significant, although much weaker, extent than an equimolar dose of MET. Since a co-treatment with MET plus TAU lowered the plasma glucose to about the same extent as MET alone, it is possible that the contribution of TAU to glucose homeostasis in the presence of MET is either overshadowed by that of MET when both compounds are administered concurrently and in equimolar doses or that they may be sharing similar modes of action. In contrast, a parallel treatment with INS led to a plasma glucose level that was rather similar to the control glucose value.

MET and TAU were also found to positively influence the plasma INS level, which was markedly decreased in diabetic rats. As was the case with the diabetic plasma glucose levels, MET was much more potent than TAU in raising the INS

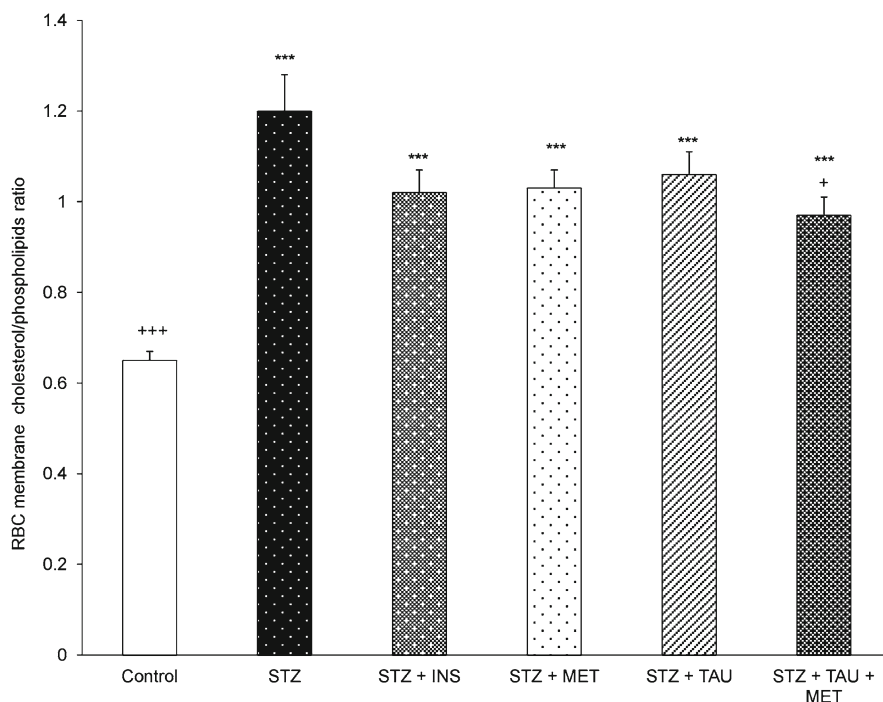

Fig. 10 The effect of a 6 week daily treatment with MET, TAU and MET-TAU, started at 14 days after the induction of diabetes with STZ, on the diabetic RBC membrane cholesterol/phospholipids ratio on day 56. INS served as a reference treatment. Data are shown as mean ± SEM for n = 6. Differences were significant vs. control at $^{***}p<0.001$; and vs. STZ at $^{+}p<0.05$ and $^{+++}p<0.001$.

levels in diabetic rats. On the other hand, a combined treatment with MET plus TAU resulted in an only a small increase in plasma INS relative to the level observed in the presence of either MET or TAU.

To obtain an estimate of the effectiveness of MET and TAU in controlling the plasma glucose levels over a 6 week period the concentration of blood HbA_{1c} was measured at the end the treatment periods. MET was found to lower the increase in blood HbA_{1c} caused by diabetes to a value not significantly different from that of naive rats and to an extent of the same magnitude as that by INS., TAU also demonstrated a significant lowering effect on the diabetic HbA_{1c} concentration but which was weaker than that of either MET or INS. In general, the effects of monotherapy with MET or TAU and with INS correlated closely with those on the plasma glucose and INS levels. On the other hand, a concurrent treatment of the diabetic rats with MET plus TAU reduced the blood HbA_{1c} to a similar extent as MET alone.

The role of MET and TAU in diabetes-induced oxidative stress was investigated by measuring the extent of LPO, changes in the GSH redox status, and changes in the activities of antioxidant enzymes both in the plasma and RBCs. In addition, the

possibility of cell membrane damage as a result of oxidative stress was examined by measuring the extent of hemolysis. The formation of MDA, serving as an indicator of end-stage LPO, was clearly increased both in the plasma and, to a lesser extent, in the RBCs of diabetic rats. MET and TAU were highly protective against these increases, with the values not differing significantly from those of control rats and of INS-treated rats. A treatment with MET plus TAU led to a protection similar to that attained with either MET or TAU. Similarly, MET and TAU were found to limit the decreases in plasma and RBC levels of GSH and accompanying increases in GSSG levels. As a result, the GSH/GSSG ratios were much lower in the diabetic than in control rats, with the decrease being more pronounced in the plasma than in the RBC. All three treatment agents were very effective in limiting the diabetic changes in GSH and GSSG levels and, thereby, in GSH/GSSG ratios, with TAU providing a greater protection than INS and MET, in that order, in the plasma, and the three agents appearing about equipotent in the RBC. The effect of a combined treatment with MET plus TAU was more protective than either agent alone in the plasma and insignificantly better in the RBC. Interestingly, while the values of plasma GSH/GSSG ratio fluctuated within a relatively wide range, those in the RBCs were rather similar and not differing from the control values as much as the plasma levels. Since the level of MDA is reported to increase only after most of the RBC GSH content has been oxidized by peroxides, the protection afforded by MET, TAU or their combination against LPO may stem from their ability to maintain the GSH pool by protecting the activities of enzymes relevant to GSH synthesis such as glutathione reductase, GPx, and glutathione cysteine synthase (Acharya and Lau-Cam 2010; Ramachandran and Saraswathy 2014).

Oxidative stress is also known to affect the intracellular and circulating antioxidant defenses. In agreement with the results of Dave and Kalia (2007) in the present work, diabetes was found to cause a profound reduction in the activities of CAT, GPx and SOD in the plasma and RBCs. These reductions, possibly the result of oxidative modification and damage (Tabatabaie and Floyd 1994), were effectively antagonized by a treatment with either MET or TAU, with MET providing a greater protection than TAU for GPx, and TAU appearing more effective than MET for CAT and SOD. In each instance, a treatment with MET plus TAU resulted in an insignificant potency gain relative to the potency seen when used individually. INS was also very protective but the extent of the protection varied according to the particular enzyme. Thus, it was more potent than either MET or TAU in the case of SOD, was equipotent with MET-TAU in protecting CAT, and was intermediate in potency to MET and TAU in the sparing GPx.

Changes in RBC membrane composition were examined by measuring the concentrations of membrane cholesterol and phospholipids. In diabetic RBCs, there was a significant increase in both the membrane cholesterol and phospholipid content, with the extent of the cholesterol increase exceeding that of the phospholipids. As a result, the membrane cholesterol to phospholipid ratio was much higher than normal. An increase in membrane cholesterol has been previously correlated with a decrease in the fluidity of the RBC membrane in comparison to control membranes (Pytel et al. 2013). Both MET and TAU were equipotent in

lowering the increases in RBC membrane lipids, especially that of cholesterol. A co-treatment with MET plus TAU was more protective than the individual treatments, and one with INS was as potent as MET or TAU in lowering the cholesterol to phospholipids ratio.

The present results give credence to the notion that TAU is an antioxidant compound in spite of its lack of a readily oxidizable functional group or that will allow to effectively scavenge oxygen derived free radicals in cell-free free radical generating systems (Aruoma et al. 1988; Tadolini et al. 1995). It would appear that TAU exerts its antioxidant effects in RBCs in an indirect manner, possibly by scavenging activated carbonyl such as MDA (Deng et al. 2013), by suppressing ROS formation and providing intracellular Ca^{2+} stabilization (Derlacz et al. 2007; di Wu et al. 1999) or by protecting mechanisms that replenish the intracellular stores of GSH during oxidative disturbances (Derlacz et al. 2007; Yu and Kim 2009). Alternatively, TAU may attenuate oxidative stress by preserving the pool of intracellular GSH (Issabeagloo et al. 2011) and the activities of enzymes degrading cytotoxic ROS (Acharya and Lau-Cam 2010; Hahn et al. 2001; Issabeagloo et al. 2011; Pushpakiran et al. 2004) or contributing to the synthesis of GSH (Acharya and Lau-Cam 2010). On the other hand, MET is a first line antidiabetic drug which, in addition to a hypoglycemic action, has also demonstrated antioxidant properties of benefit in diabetes. Besides acting as a free radical scavenger and as modulator of intracellular production of superoxide anion (Bonnefont-Rousselot et al. 2003), this biguanide has also been found to inhibit xanthine oxidase activity and AGEs formation, to show a chelating action on metal ions mediating radical formation (Zatalia and Sanusi 2013), to restore the antioxidant status and activities of antioxidant enzymes, and to enhance the total thiol and nitric oxide levels in type 2 diabetic patients (Chakraborty et al. 2011). In rats treated with STZ, MET was also found to restore the expression of antioxidant genes found to suppress hyperglycemia-induced production of ROS in the kidney and microvascular endothelium (Alhaider et al. 2011); increase intracellular GSH levels in blood and liver of diabetic rats (Ewis and Abdel-Rahman 1995; Ramachandran and Saraswathy 2014; Yanardag et al. 2005).

5 Conclusions

The present results confirm the damaging and altering actions of oxidative stress on the RBC membrane as inferred from the presence of extensive hemolysis, the development of LPO, and changes in cholesterol and phospholipids levels. The damaging actions of oxidative stress are found to positively correlate with prolonged hyperglycemia, an increase in Hbglycation, and decreases in enzymatic and nonenzymatic antioxidant defenses. Furthermore, a treatment of diabetic rats with either MET or TAU is found to be highly protective against the alterations associated with diabetes. Based on the intrinsic properties of these compounds germane to oxidative stress, it appears that hyperglycemia and oxidative stress may be acting, at least in part, independently since TAU demonstrated a high protective effect without a

commensurable significant effect on the plasma glucose or INS levels as was the case with MET. This conclusion is confirmed by the greater antioxidant action of a co-treatment with MET and TAU than with either compound alone, in all likelihood due to their complementary actions. Generally, MET plus TAU provided an antioxidant action that about equipotent with that of INS.

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Taurine Ameliorates Hypercholesterolemia But Not Obesity in Rats Fed a Lard-Based, High-Fat Diet

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Abbreviations

LDL-C	Low-density lipoprotein cholesterol
HDL-C	High-density lipoprotein cholesterol
Tau	Taurine
CVD	Cardiovascular disease
HF	High fat
TC	Total cholesterol

1 Introduction

Excessive caloric intake and a sedentary lifestyle have rapidly increased the prevalence of obesity and its associated comorbidities, such as hyperlipidemia, metabolic syndrome, and type 2 diabetes, worldwide (Angulo 2002). In particular, hyperlipidemia, which is characterized by high levels of blood total cholesterol (TC),

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low-density lipoprotein cholesterol (LDL-C), triglycerides (TG), and low levels of high-density lipoprotein cholesterol (HDL-C), is considered one of the major risk factors for cardiovascular disease (CVD), which is a leading cause of worldwide morbidity and mortality (Kostis 2007). It is projected that, by 2020, CVD will surpass infectious disease as the world's leading cause of death and disability (Levenson et al. 2002). However, the incidence of CVD is significantly higher in Western countries than in Asian countries (Adlercreutz 1990). One explanation for Asia's lower CVD incidence is the higher proportion of fish and lower intake of meat and fat in Asian diets compared to Western populations.

Taurine, which is abundant in fish and shellfish, has been found to lower serum cholesterol levels and suppress the development of atherosclerosis in experimental animals (Matsushima et al. 2003; Murakami et al. 1999; Petty et al. 1990). Moreover, taurine has been shown to decrease fat accumulation in obese mice (Mikami et al. 2012); thus, diets deficient in taurine may promote obesity (Tsuboyama-Kasaoka et al. 2006). In previous research, we showed that taurine lowered serum lipid levels in Otsuka Long-Evans Tokushima Fatty (OLETF) rats that were fed a normal diet (Kim et al. 2012).

The cholesterol lowering effects of taurine have been consistently observed in rats and mice with exogenous hypercholesterolemia caused by a diet high in cholesterol/sodium and cholate (Chen et al. 2012). Cholate is generally added to the diet to induce hypercholesterolemia in rats because it enhances cholesterol uptake. Meanwhile, the cholesterol lowering effects of taurine differ depending on the species and the type of hypercholesterolemia. In particular, taurine's effect seems to be different in hypercholesterolemia cases that were induced by a high-cholesterol diet without sodium cholate. One reason is that taurine only induces cholesterol 7a-hydroxylase (CYP7A1) mRNA in the presence of cholate in a high-cholesterol diet (Chen et al. 2005). Furthermore, taurine did not reduce serum and liver total cholesterol concentrations in cases of endogenous hypercholesterolemia that were induced by phenobarbital (PB) or polychlorinated biphenyl (PCB) (Chen et al. 2012). Thus, it is better to test the cholesterol lowering effect of taurine on hypercholesterolemia in an obese animal model with a high-fat diet, rather than a high cholesterol/sodium diet with cholate. Additionally, a lard-based high-fat (HF) diet seems to be more similar to the diets of obese patients than is a high cholesterol/sodium diet with cholate. In this study, we tested the cholesterol lowering effects and anti-obesity effects of taurine in rats fed a lard-based, high-fat diet.

2 Methods

2.1 Preparation of Animals and Diets

Four-week old male Sprague-Dawley rats were purchased from Koatech (Anseong, Korea). All rats were kept in laboratory animal housing at Kyung Hee University, following recommendations from the Guide for the Care and Use of Laboratory

Table 1 Composition of experimental diet

Component	Normal diet (g)	High-fat diet (g)
Casein	200.0	200.0
Corn starch	447.486	155.036
Sucrose	50.0	50.0
Dextrose	132.0	132.0
Cellulose	50.0	50.0
Vitamin mixture[a]	10.0	10.0
Mineral mixture[b]	35.0	35.0
Soybean oil	70.0	25.0
Lard	–	175.0
L-cystine	3.0	3.0
Choline bitartrate	2.5	2.5
TBHQ	0.014	0.014

[a]AIN-93 vitamin mixture

[b]AIN-93 mineral mixture

Animals with a constant 12-h light and dark cycle (07:00 am–07:00 pm), controlled temperature (22 ± 1 °C), and humidity (55 ± 10 %). Following 1 week of acclimatization with a pelletized commercial diet, the rats were randomly divided into four diet groups: normal diet (N group), high-fat diet (HF group), HF diet for 6 weeks followed by taurine diet for 6 weeks (Tau 6 group), taurine diet for 12 weeks (Tau 12 group). The taurine diet was prepared by adding taurine (2 %) to the HF diet. Food and water intakes were measured twice per week and body weight was measured once per week. The composition of the experimental diet was based on AIN-93 (Reeves 1997), as shown in Table 1.

2.2 Sampling and Chemical Analysis

After 12 weeks, the animals were fasted for 12 h before sacrifice. Blood was collected from the heart and blood serum was obtained by centrifugation at 3,000 rpm for 20 min. The serum was stored at −70 °C until analysis. Serum total glyceride (TG) and total cholesterol (TC) levels were analyzed using an automatic analyzer (BPC BioSed, Rome, Italy). High-density lipoprotein-cholesterol (HDL-C) was obtained from whole serum with a high-density lipoprotein precipitation reagent (Asan Pharm, Seoul, Korea) after precipitation of low-density lipoproteins and very-low-density lipoproteins for 10 min at 3,000 rpm. They were then analyzed for HDL-C using the same method as with TC. The serum low-density lipoprotein cholesterol (LDL-C) value was calculated using the Friedewald formula (Friedewald et al. 1972): LDL-C = TC-(HDL-C + TG/5).

Assayed chemistry control (Bio-Rad Laboratories, Hercules, CA, USA) was used for calibration. All results are expressed as mg/dl of serum.

Analysis of serum adiponectin levels were performed using the MILLIPLEX MAP Rat Single Plex Adiponectin 96-well Plate kit (EMD Millipore, Billerica, MA, USA) and analysis of serum leptin levels was performed using the MILLIPLEX MAP Rat Serum Adipokine 96-well Plate kit (EMD Millipore) with the Luminex 100 (Hitachi, Tokyo, Japan).

2.3 Statistic Analysis

Data are expressed as the mean ± standard error of the mean (SEM) and were analyzed for significant differences between diet group by one-way analysis of variance (ANOVA), followed by Duncan's multiple range tests; significance was set at $p < 0.05$. All analyses were performed using SPSS, version 17.0 (IBM, Armonk, NY, USA).

3 Results

3.1 Effect of Taurine on Body Weight and Fat-Tissue Weight After Receiving a High-Fat Diet

First, to test if taurine affects the body weight among rats fed a lard-based, high-fat diet, the rats were divided into four groups according to diet, as described above. As shown in Table 2, body weight gain (g/day) in the HF group increased significantly compared to that of N group. However, the taurine-diet groups (Tau 6 and Tau 12), did not experience a reduction in body weight after feeding for 6 or 12 weeks, even though there was no significant difference in food intake (kcal/day) between groups.

Table 2 Effect of taurine on body weight gain, diet intake, water intake and food efficiency ratio in rats fed a high-fat diet

Group	N	HF	Tau 6	Tau 12
IBW	69.41 ± 2.17	69.34 ± 2.03	69.57 ± 2.23	69.48 ± 2.25
BWG	1.23 ± 0.13[a]	1.91 ± 0.13[b]	2.15 ± 0.13[b]	2.12 ± 0.12[b]
FI	16.05 ± 0.38[a]	12.54 ± 0.53[b]	12.91 ± 0.61[b]	12.38 ± 0.61[b]
FI*	66.34 ± 1.61	59.94 ± 2.51	61.75 ± 2.92	59.21 ± 2.90
WI	21.36 ± 0.44	22.68 ± 0.82	23.81 ± 0.50	22.48 ± 0.63
FER	0.08 ± 0.01[a]	0.15 ± 0.01[b]	0.16 ± 0.01[b]	0.17 ± 0.01[b]

Values are expressed as mean ± SEM. Values with *different superscripts* within the row are significantly different at $p < 0.05$ by Duncan's multiple range test. *N* normal diet group, *HF* high-fat diet group, *Tau 6* HF diet for 6 weeks followed by taurine diet for 6 weeks, *Tau 12* taurine diet for 12 weeks. The food efficiency ratio is the daily weight gain per daily dietary intake. *IBW* initial body weight, *BWG* body weight gain (g/day), *FI* food intake (g/day), *FI** food intake (kcal/day), *WI* water intake (g/day), *FER* food efficiency ratio

Table 3 Effect of taurine on relative organ weights in rats fed a high-fat diet

Group	N	HF	Tau 6	Tau 12
Liver	2.11 ± 0.04^{a}	2.00 ± 0.04^{b}	1.94 ± 0.03^{b}	1.89 ± 0.04^{b}
Spleen	0.18 ± 0.01	0.18 ± 0.01	0.17 ± 0.01	0.16 ± 0.01
Kidney	0.61 ± 0.02^{a}	$0.57 \pm 0.01^{a,b}$	$0.57 \pm 0.02^{a,b}$	0.53 ± 0.01^{b}
Epididymal fat	1.10 ± 0.05^{a}	1.64 ± 0.09^{b}	1.63 ± 0.07^{b}	1.65 ± 0.10^{b}
Retroperitoneal fat	0.98 ± 0.09^{a}	1.63 ± 0.13^{b}	1.78 ± 0.09^{b}	1.71 ± 0.15^{b}

Values are expressed as mean ± SEM. Values with *different superscripts* within the row are significantly different at $p < 0.05$ by Duncan's multiple range test. *BW* body weight, *N* normal diet group, *HF* high-fat diet group, *T6* HF diet for 6 weeks followed by taurine diet for 6 weeks, *T12* taurine diet for 12 weeks. Organ weight unit: g/100 g BW

On the other hand, we checked if taurine supplementation reduced the organ weight gain that occurred as a result of a high-fat diet. As shown in Table 3, the weight of epididymal and retroperitoneal fat tissue in rats in the HF group increased significantly compared to that of the N group, while taurine supplementation did not result in weight loss that was significantly different from the final weight of the HF group. The weight of the liver, spleen and kidneys were also not affected by taurine supplementation. Rather, a combination of a high-fat and taurine diet may slightly decrease the weight of the three organs compared to that of the N group. Additionally, significant histological differences between the pancreas, liver, and adipose tissue were not detected between the three groups (data not shown). All these results suggest that taurine supplementation is not effective for decreasing weight gain in a rat model where obesity was induced by a lard-based, high-fat diet.

3.2 *Effect of Taurine on Serum Lipid Profiles in Rats Fed a High-Fat Diet*

To test if hyperlipidemia, which was induced by a lard-based, high-fat diet, was ameliorated by dietary taurine, we collected blood sera from all rats at the end of their 12-week feeding regimen. As shown in Table 4, the serum levels of the HF group compared to the N group were significantly higher for triglyceride (51.31 ± 3.13 vs. 39.07 ± 1.39 mg/dl), total cholesterol (93.68 ± 2.52 vs. 84.21 ± 3.76 mg/dl), and LDL-cholesterol (52.32 ± 1.98 vs. 41.62 ± 3.05 mg/dl). However, the serum lipid levels in the HF group decreased significantly among the rats in the taurine groups. The triglyceride levels (36.24 ± 1.10 vs. 51.31 ± 3.13 mg/dl), total cholesterol levels (78.28 ± 2.89 vs. 93.68 ± 2.52 mg/dl), and LDL-cholesterol levels (46.25 ± 2.10 vs. 52.32 ± 1.98 mg/dl) in the Tau groups were lower than that of the N group after 12 weeks. In contrast, the serum HDL-cholesterol levels in the HF group did not increase over the 12 weeks, but those levels decreased significantly in the Tau group compared to that of the N group.

Table 4 Effect of taurine on serum lipid profiles in rats fed a high-fat diet

Group	N	HF	Tau 6	Tau 12
Glyceride	39.07 ± 1.39[a]	51.31 ± 3.13[b]	37.58 ± 2.04	36.24 ± 1.10[a]
TC	84.21 ± 3.76[a]	84.21 ± 3.76[a]	86.23 ± 1.94	78.28 ± 2.89[a]
LDL-C	41.62 ± 3.05[a]	52.32 ± 1.98[b]	49.98 ± 2.22[b]	46.25 ± 2.10[a,b]
HDL-C	31.83 ± 1.66	30.75 ± 1.54[a]	28.73 ± 1.75[a,b]	24.78 ± 1.32[b]

Values are expressed as mean ± SEM. Values with *different superscripts* within the row are significantly different at $p < 0.05$ by Duncan's multiple range test. *N* normal diet group, *HF* high-fat diet group. Serum lipid level unit: mg/dl

4 Discussion

In this study, the cholesterol lowering and anti-obesity effects of taurine were tested in a rat model that was fed a lard-based, high-fat diet. In the obese rat model, taurine supplementation was effective at lowering the serum lipid levels, but not at reversing weight gain. In cases of hypercholesterolemia induced by a high cholesterol/sodium and cholate diet, there are three possible metabolic and molecular mechanisms of taurine's cholesterol lowering effect: cholesterol clearance from blood circulation, bioconversion of cholesterol to bile acid in the liver, and excretion of cholesterol and bile acid from the intestines (Chen et al. 2012). In this model, increased serum cholesterol and lipid levels seemed to have decreased significantly in the presence of taurine through the above three mechanisms. However, taurine supplementation did not reduce the weight of epididymal and retroperitoneal fat tissue, nor did it decrease the overall body weight gain (g/day) that was induced by a high-fat diet, even though taurine supplementation did significantly reduce body weight in an obese experimental animal model (Zhang et al. 2004). The cholesterol-lowering and lipid-lowering effects of taurine have been consistently observed in many other experimental animals, but taurine's anti-obesity properties are less convincing. These contradictory results may be due to methodological differences, including different dosages used in different studies and variations in supplementation periods. On the other hand, the cholesterol lowering effects could differ according to the type of dietary fatty acids fed to ovariectomized rats. Taurine lowered the plasma total cholesterol concentration in rats that were fed corn oil, but not rats fed coconut oil (Kishida et al. 2003). In this study, liver total lipid levels were increased by taurine supplementation, while plasma lipids decreased. This partly suggests that blood lipid returns to the liver more frequently in the presence of taurine, which increases the expression of LDL-R in the liver.

Hyperlipidemia is a well-known risk factor of cardiovascular disease, and high blood LDL-cholesterol (LDL-C) may be the most important risk factor. HDL-cholesterol (HDL-C) mediates reverse cholesterol transport and exhibits numerous beneficial properties, including antioxidant, anti-inflammatory, and antithrombotic effects on the vasculature (Young et al. 2004). In this model, we could not properly detect the effect of taurine on HDL-C levels because the rats fed a high-fat diet did not show any significant decrease in their serum HDL-C levels. Nevertheless,

taurine supplementation significantly decreased serum LDL-C levels, which suggests that taurine decreased LDL-C and triglyceride serum levels through a mechanism that is independent of HDL-C-related mechanisms. The anti-atherosclerotic effects of taurine have been shown across animal species, with some evidence for an effect in humans as well (Matsushima et al. 2003). Therefore, it has been suggested that a combination therapy including a taurine diet and a statin may be appropriate for certain hyperlipidemia patients because statin therapy alone may increase the risk of myopathy and diabetes (Ito 2012).

5 Conclusion

Taurine supplementation is highly effective at lowering the serum triglyceride and LDL-C levels when they have been increased by a lard-based, high-fat diet in animal models. Therefore, taurine supplementation may be helpful in the amelioration of hyperlipidemia, a common condition in obese patients and one of the major risk factors of cardiovascular disease (CVD).

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Impact of Light Ethanol Intake and of Taurine, Separately and Together, on Pathways of Glucose Metabolism in the Kidney of Diabetic Rats

Sanket N. Patel, Mitul Parikh, and Cesar A. Lau-Cam

Abbreviations

STZ	Streptozotocin
TAU	Taurine
EtOH	Ethanol
HK	Hexokinase
GKL	Glucokinase-like
PFK-1	Phosphofructokinase-1
PK	Pyruvate kinase
GAPDH	Glyceraldehydes-3-phosphate dehydrogenase
PC	Pyruvate carboxylase
cPEPCK	Cytosolic phosphoenolpyruvate carboxykinase
FBPase	Fructose-1,-6-bisphosphatase
G6Pase	Glucose 6-phosphatase
TCA	Tricarboxylic acid cycle
CS	Citrate synthase
cMDH	Cytosolic malate dehydrogenase
G6PDH	Glucose 6-phosphate dehydrogenase
6PGDH	6-Phosphogluconate dehydrogenase
AR	Aldose reductase
SDH	Sorbitol dehydrogenase

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1 Introduction

Diabetic nephropathy is a serious microvascular complication of diabetes mellitus characterized by anatomical, structural and functional changes leading to thickening of the glomerular basement membrane, microaneurysm, damaged small blood vessels, organ dysfunction, renal failure and even death (Fowler 2008). The development and progression of these diabetic complication is closely associated with chronic hyperglycemia, which is regarded as the driving force of diabetic vascular complications through a multitude of metabolic and structural derangements, including the production of reactive oxygen and nitrogen species, and advanced glycated end products, overexpression of several growth factors, and the activation of signaling cascades and inflammatory cells (Cade 2008; Galkina and Ley 2006). Additional common risk factors are insulin resistance, dyslipidemia, hypertension, obesity, smoking, dietary factors (Cade 2008; Gross et al. 2005) and a familial predisposition (Tarnow et al. 2000).

One of the distinguishing metabolic features of patients with type 2 diabetes is increased plasma glucose levels with accompanying insulin levels that may not be grossly different from those seen in healthy individuals. This finding suggests the existence of altered insulin function (or insulin resistance) in suppressing endogenous glucose production, mainly via gluconeogenesis and secondarily via glycogenesis, and utilization as a result of the activity of glycolysis-tricarboxylic acid pathway (TCA), the pentose phosphate pathway (PPP) and the sorbitol (polyol) pathway (Bonadonna 2004; Kinoshita 1965). In addition, and depending on the tissue, diabetes can stimulate or inhibit the activity of malate dehydrogenase (MDH) to influence the malate-aspartate shuttle and, thereby, the activity of glycolysis and the transfer of cytosolic reducing equivalents from NADH to the respiratory chain for oxidation, under diabetic conditions (Kazmi et al. 1985; Puckett and Reddy 1979; Safer 1975).

Ethanol (EtOH) is known to influence glucose metabolism in both healthy (Yki-Järvinen and Nikkilä 1985) and diabetic patients (van der Wiel 2004), with the effects depending on the amount and duration of the consumption. While chronic alcoholics have been found to exhibit an increased baseline glucose production rate, insulin resistance, impaired glucose tolerance, and decreased glucose utilization (Umhau et al. 2002), low to moderate consumption may improve insulin sensitivity, limit disturbances in glycemic control, and lower the incidence of type 2 diabetes mellitus (van der Wiel 2004). There is also evidence to suggest that the acute and chronic administration of EtOH can enhance the activity of the pentose phosphate pathway (Badawy and Evans 1974; Kalant et al. 1970) and that, on an acute basis, it has little effect on the hepatic accumulation of glycogen while increasing the malate/oxaloacetate ratio (Rawat 1968). However, there is also a report indicating that a prolonged administration of EtOH (0.69 mL/100 g) to rats by gastric intubation or intraperitoneal injection resulted in total abolition of glycogen synthesis in oxidative muscle in response to insulin (Xu et al. 1992, 1996). Decreased hepatic glycogen levels after EtOH infusion in spite of a normal blood glucose level has also

been verified in rats (Clark and Evans 1960). In common with glycolysis, EtOH also depresses the activity of the TCA cycle, evident from a decrease in carbon dioxide formation, and promotes the accumulation of acetyl CoA (Hawkins and Kalant 1972). Moreover, in chronic alcoholics, sorbitol oxidation and disappearance from the circulation is found to be impaired (Badawy 1977); and the intake of a 48 g dose of EtOH by overnight fasted normal men is reported to lower hepatic gluconeogenesis, glycogenolysis, glucose, and the availability of intracellular precursors of gluconeogenesis (Siler et al. 1998).

Taurine (TAU) is nonprotein β-amino acid endowed with a wide range of biological actions, including an effect on glucose homeostasis and islet function. In mice consuming TAU as 2 % supplement to the diet (Carneiro et al. 2009) or as an addition to the drinking water (Ribeiro et al. 2009) there was an increase in insulin secretion, in the sensitivity of islet cells to stimulatory concentrations of glucose, in glucose metabolism and in glucose tolerance along with stabilization of cytosolic fluctuations in Ca^{2+} levels compared to control mice. In addition islet cells isolated from the mice secreted more insulin when challenged with high concentrations of glucose and L-leucine (Ribeiro et al. 2009). TAU has also been found to enhance peripheral insulin sensitivity in response to hyperglycemia (Nakaya et al. 2000), to potentiate insulin-mediated glucose uptake, and to stimulate glycolysis, glycogenesis and oxygen utilization (Lampson et al. 1983). In streptozotocin-treated diabetic rats supplementation of the diet with TAU was shown to prevent the loss of insulin and to stimulate adrenoreceptor-stimulated glucose uptake into adipocytes (Colivicchi et al. 2004). Although TAU can enhance (Ribeiro et al. 2009) or restore glucose-stimulated (Batista et al. 2012) insulin secretion, a daily supplementation with this amino acid (1.5 g for 8 weeks) was without effect on insulin secretion or sensitivity and on blood lipid levels of nondiabetic overweight men with a familial history of type 2 diabetes mellitus (Brøns et al. 2004). A lack of a consistent effect of a TAU supplementation on the hyperglycemia of diabetes has also been observed in humans and rodents. While in one study TAU was found to induce a marked decreased of the circulating glucose level in type 1 diabetic patients (Elizarova and Nedosugova 1996), in another, carried out with type 2 diabetics, it showed no significant effect (Chauncey et al. 2003). Similarly, TAU is reported to lower hyperglycemia, to improve insulin sensitivity and to lower serum cholesterol and triacylglycerols in the Otsuka Long-Evans Tokushima Fatty rat, an animal model of diabetes with obesity (Nakaya et al. 2000); but in Goto-Kakizaki rats, known to develop type 2 diabetes spontaneously, feeding a cholesterol-free diet supplemented with 3 % TAU resulted in no effect on the plasma cholesterol and insulin, and in an increase in plasma HDL-cholesterol (Nishimura et al. 2002).

Based on the known actions of EtOH and TAU on glucose metabolism in clinical and experimental diabetes, the present study was undertaken in diabetic rats to specifically determine whether a low intake of EtOH or an oral treatment with TAU can influence the changes in glucose metabolism by classical and alternative pathways caused by diabetes, and whether their individual effects can be enhanced when they are made available concurrently.

2 Methods

2.1 Animals, Treatments and Samples

The experiments were conducted using male Sprague-Dawley rats, 310–340 g in weight, assigned to groups of six. The solution of EtOH (5 % in tap water, v/v, sweetened with Splenda® and flavored with a commercial sugar-free powdered lemonade product) was prepared from a 200 proof sample, and was available in place of the drinking water from days 1 to 28. Diabetes was induced on day 15 with streptozotocin (STZ), 60 mg/kg i.p. in 10 mM citrate buffer pH 4.5. TAU (2.4 mM/kg) was provided by oral gavage, with or without the concurrent availability of EtOH. Rats drinking only tap water or only 5 % EtOH or receiving only STZ or only TAU served as controls. All the animals were sacrificed on day 29 by cardiac puncture under isoflurane anesthesia, and their kidneys were collected without delay using the freeze clamp technique. A portion of each kidney (~300 mg) was made into a 1:20 (w/v) homogenate in a solution of 250 mM sucrose, 1 mM glutathione, 1 mM EDTA, 5 mM cysteine and 1 mM dithiothreitol, and the resulting suspension was centrifuged at 2,000 × *g* and 4 °C for 30 min to obtain a clear supernatant. The supernatant was used for the assay of glucose, glycogen and enzymes present in the soluble fraction (except PC and CS). The pellet was resuspended in 1 mL of PBS, pH 7.4, dispersed with the help of a microspatula, and subjected two cycles of sonication. The suspension was centrifuged at 10,000 × *g* for 30 min, and the supernatant was used for the assay of PC and CS. The blood samples, collected in heparinized tubes, were processed for their plasma fractions, which were used for the assay of glucose. The study was approved by the Institutional Animal Care and Use Committee of St. John's University, Jamaica, NY, and the animals were cared in accordance with guidelines established by the United States Department of Agriculture. The study was approved by the Institutional Animal Care and Use Committee of St. John's University, Jamaica, NY, and the animals were cared in accordance with guidelines established by the United States Department of Agriculture.

2.2 Biochemical Assays

The kidney homogenates were assayed for the activity of enzymes related to glycolysis (hexokinase-1 = HK-1, glucokinase-like = GKL, phosphofructo-kinase-1 = PFK-1, pyruvate kinase = PK, glyceraldehyde 3-phosphate dehydrogenase = GAPDH), gluconeogenesis (pyruvate carboxylase = PC, cytosolic phosphoenolpyruvate carboxykinase = cPEPCK, fructose 1,6-bisphosphatase = FBPase, glucose 6-phosphatase = G6Pase), TCA cycle (citrate synthase = CS), malate shuttle (cytosolic malate dehydrogenase = cMDH), pentose phosphate pathway (glucose 6-phosphate dehydrogenase = G6PDH, 6-phosphogluconate dehydrogenase = 6PGDH)

and polyol pathway (aldose reductase = AR, sorbitol dehydrogenase = SDH) and for their glucose and glycogen contents. The glycolytic enzymes HK-1, GKL, PFK-1, PK and GAPDH were measured as described by Shonk and Boxer (1964). Gluconeogenic enzymes were measured using published methods, i.e., PC according to Duruibe and Tejwani (1981), cPEPCK according to Chang and Lane (1966), FBPase according to Taketa and Pogell (1965), and G6Pase according to Gierow and Jergil (1980). The activity of CS was measured according to Srere (1959) and that of cMDH according to Bergmeyer (1983a). The activity of the pentose phosphate pathway was assessed on the basis of the activities of G6PDH and 6PGDH, which were measured as described by Bergmeyer (1983b) and Bergmeyer (1983c), respectively. AR and SDH were measured according to Chang et al. (2002) and Bergmeyer (1983d). Except for the results of FBPase, which were expressed in μM product formed/μg of protein/min, those of the other enzymes were expressed in μM of product formed/mg of protein/min. The content of kidney glycogen, in μg/g of kidney, was measured in a 1:20 (w/v) homogenate in 0.6 N perchloric using the method of Keppler and Decker (1974). The plasma and renal glucose concentrations were measured using a commercial enzymatic assay kit based on the method of Raabo and Terkildsen (1960) (Procedure No. 510, Sigma-Aldrich, St. Louis, MO), and the results are reported as mg/dL and μg/mg of protein, respectively.

2.3 Statistical Analysis of the Data

The results, reported as mean ± standard error of the mean (SEM) for $n = 6$ rats, were analyzed for statistical significance by unpaired Student's *t*-test, followed by one-way analysis of variance (ANOVA) and Tukey's *post hoc* test. Intergroup differences were considered to be significant when p was ≤ 0.05.

3 Results

3.1 Plasma Glucose

As shown in Fig. 1, diabetic rats exhibited plasma glucose levels that were much higher (by ~340 %, $p < 0.001$) at 2 weeks after the induction of diabetes with STZ than nondiabetic rats of equivalent body weight and age. The same figure also shows that both EtOH and TAU were effective in lowering this increase (by 35 % and 42 %, respectively, both at $p < 0.01$ vs. control). A further lowering was observed when 5 % EtOH and TAU were provided at the same time (51 % less, $p < 0.001$ vs. diabetes). By themselves or as a combination treatment, EtOH and TAU were found to lower the plasma glucose of naive rats by about the same extent (~18 %, $p < 0.05$).

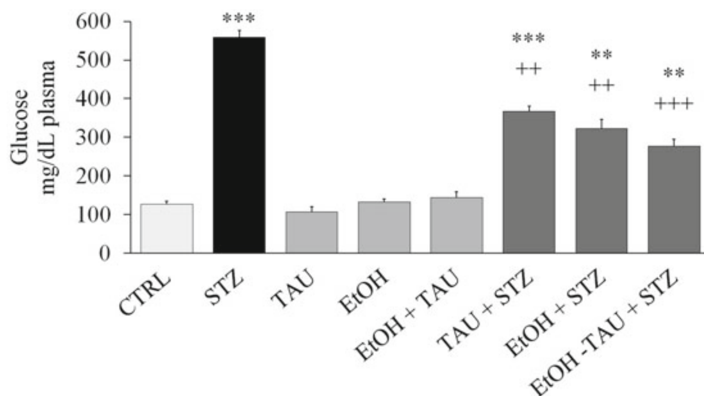

Fig. 1 Plasma glucose levels of diabetic (STZ) rats in the absence and presence of 5 % EtOH, TAU or 5 % EtOH plus TAU. Values are shown as mean ± SEM for $n = 6$. Differences were statistically significant vs. control at $^{**}p < 0.01$ and $^{***}p < 0.001$; and vs. STZ at $^{++}p < 0.01$ and $^{+++}p < 0.001$

3.2 Glycolytic Enzymes

The effect of diabetes on renal glycolysis was studied by measuring the activities of HK-1, GKL, PFK-1, GAPDH and PK. The results presented in Fig. 2a indicate that diabetes increased the activities of HK-1 (by 171 %) and GK (by eightfold) over the corresponding control values (both at $p < 0.001$). Both EtOH and TAU were very effective in reducing the diabetic increase in HK-1 activity, with TAU providing a greater attenuation than EtOH (increases of 57 % and 71 %, respectively, both at $p < 0.001$ vs. control). On the other hand a co-treatment with EtOH plus TAU was much more effective than the individual treatments (only 7 % increase). Neither the individual treatments nor the combination treatment increased the HK-1 activity of naive rats to a significant extent. Likewise, both EtOH and TAU were able to lower the increase in diabetic GKL activity, with the effect being much greater when they were used alongside (no increase) than individually (twofold increase with EtOH, threefold increase with TAU) (Fig. 2b).

As shown in Fig. 3a, b and 4, while diabetes increased the activities of PFK-1 (by 367 %, Fig. 3a) and PK (by 55 %, Fig. 3b), it decreased that of GAPDH (by 69 %, Fig. 4) relative to corresponding control values (all at $p < 0.001$). Both EtOH and TAU were able to lower the activities of PFK-1 (by 58 % and 52 %, respectively, both at $p < 0.001$, Fig. 3a) and of PK (both by 54 %, $p < 0.001$, Fig. 3b) seen in untreated diabetic rats, especially when available concurrently (70 % and 63 % decreases, respectively, $p < 0.001$ vs. diabetes). In addition, EtOH and TAU were effective in reducing the loss in GAPDH activity caused by diabetes (by 47 % and 56 %, respectively, $p < 0.001$), more as a combination treatment (only 24 % loss, $p < 0.05$) than as an individual treatment (Fig. 4). In all instances EtOH and TAU, alone or in combination, did not alter the baseline values to a significant extent (Figs. 3a, b and 4).

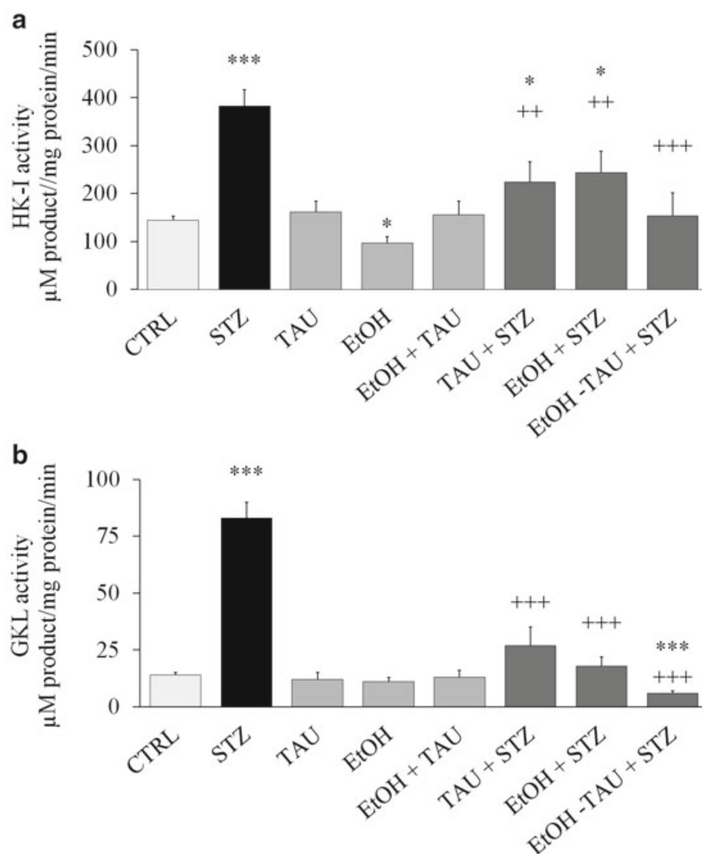

Fig. 2 Renal activities of (**a**) HK-1 and (**b**) GKL of diabetic (STZ) rats in the absence and presence of 5 % EtOH, TAU or 5 % EtOH plus TAU. Values are shown as mean ± SEM for $n = 6$. Differences were statistically significant vs. control at $^{*}p < 0.05$ and $^{***}p < 0.001$; and vs. STZ at $^{++}p < 0.01$ and $^{+++}p < 0.001$

3.3 Gluconeogenic Enzymes

The influence of diabetes on renal gluconeogenesis was examined based on the activities of PC, cPEPCK, FBPase and G6Pase in kidney homogenates. As seen in Fig. 5a, b, diabetes raised the renal activities of PC and cPEPCK, respectively. The results shown in Fig. 6a, b indicate that diabetes also raised the activities of FBPase and G6Pase, respectively, compared to corresponding control values. In common with glycolytic enzymes, these increases were effectively attenuated by both EtOH and TAU pretreatments to values in a rather uniform manner and similar extent (decreases in the range 35–41 % with EtOH and 35–42 % with TAU, all at $p < 0.01$ vs. diabetes). The availability of EtOH alongside TAU further enhanced the effect attained with the individual agents (decreases in the range 46–61 %, $p < 0.001$ vs. diabetes).

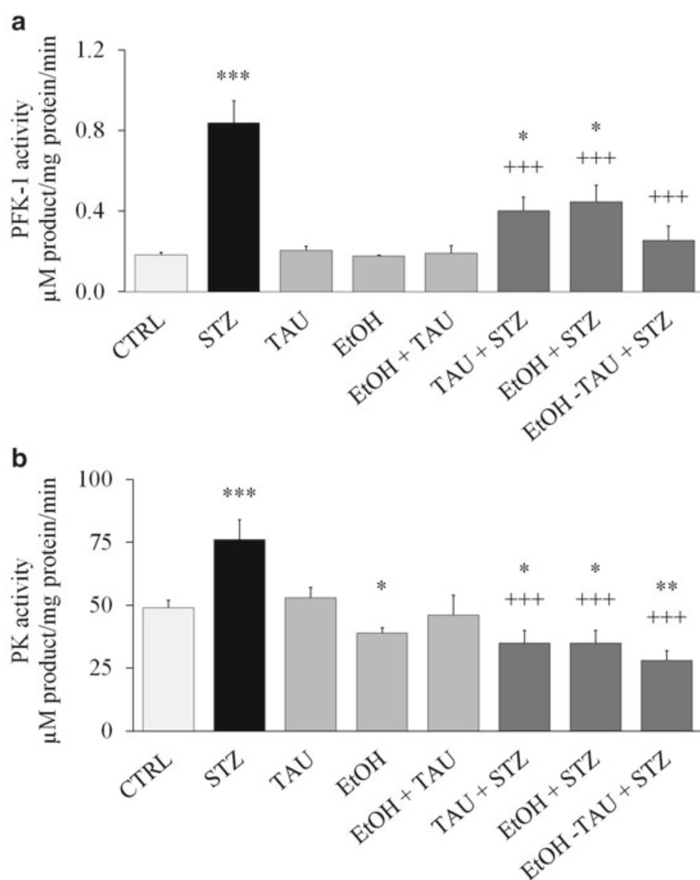

Fig. 3 Renal activities of (**a**) PFK-1 and (**b**) PK of diabetic (STZ) rats in the absence and presence of 5 % EtOH, TAU or 5 % EtOH plus TAU. Values are shown as mean ± SEM for $n = 6$. Differences were statistically significant vs. control at $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$; and vs. STZ at $^{+++}p<0.001$

3.4 Enzymes of the TCA Cycle and Malate-Aspartate Shuttle

The effect of diabetes on the renal TCA cycle was evaluated by measuring the activity of CS. The status of the transport of cytosolic reducing equivalents into the mitochondrion was defined by measuring the activity of cMDH. From the results presented in Fig. 7a, it is apparent that diabetes suppressed the activity of CS markedly (by 73 %, $p<0.001$ vs. control). This effect was attenuated by EtOH and TAU to a significant extent both when provided singly (only 42 % and 46 % decreases, respectively, $p<0.001$ vs. control) and together (only 27 % decrease, $p<0.01$ vs. control).

In diabetic rats, the renal activity of cMDH was found to increase significantly (by 128 %, $p<0.001$) relative to the control value (Fig. 7b). In contrast, the cMDH activity of diabetic rats receiving either EtOH or TAU was much lower (by 30 % and 38 %,

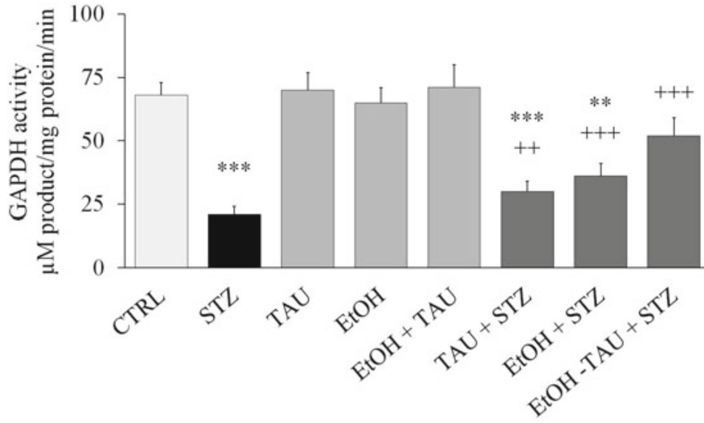

Fig. 4 Renal activity of GAPDH of diabetic (STZ) rats in the absence and presence of 5 % EtOH, TAU or 5 % EtOH plus TAU. Values are shown as mean ± SEM for $n = 6$. Differences were statistically significant vs. control at $^{**}p < 0.01$ and $^{***}p < 0.001$; and vs. STZ at $^{++}p < 0.01$ and $^{+++}p < 0.001$

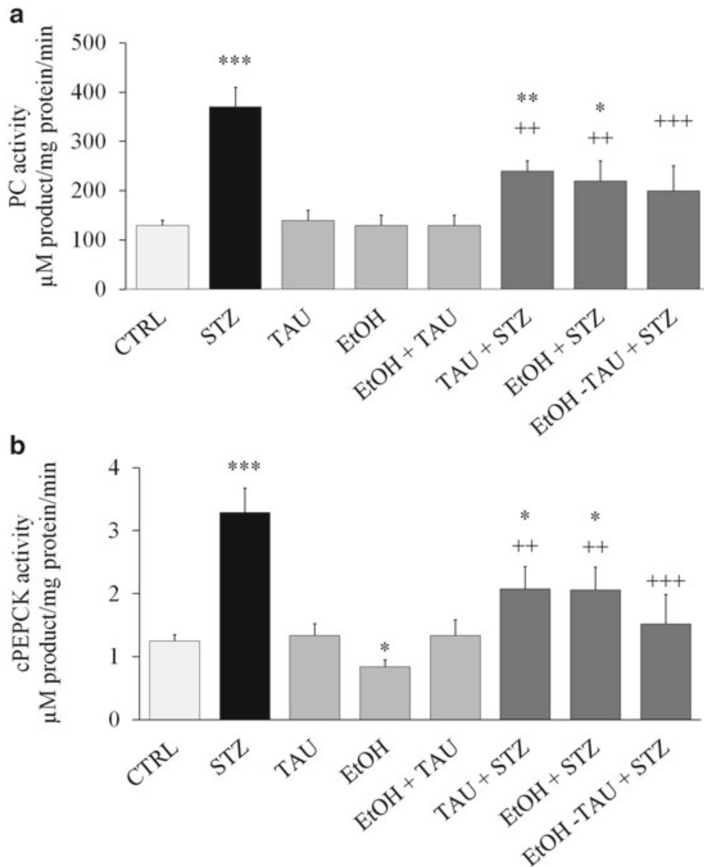

Fig. 5 Renal activities of (**a**) PC and (**b**) cPEPCK of diabetic (STZ) rats in the absence and presence of 5 % EtOH, TAU or 5 % EtOH plus TAU. Values are shown as mean ± SEM for $n = 6$. Differences were statistically significant vs. control at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$; and vs. STZ at $^{++}p < 0.01$ and $^{+++}p < 0.001$

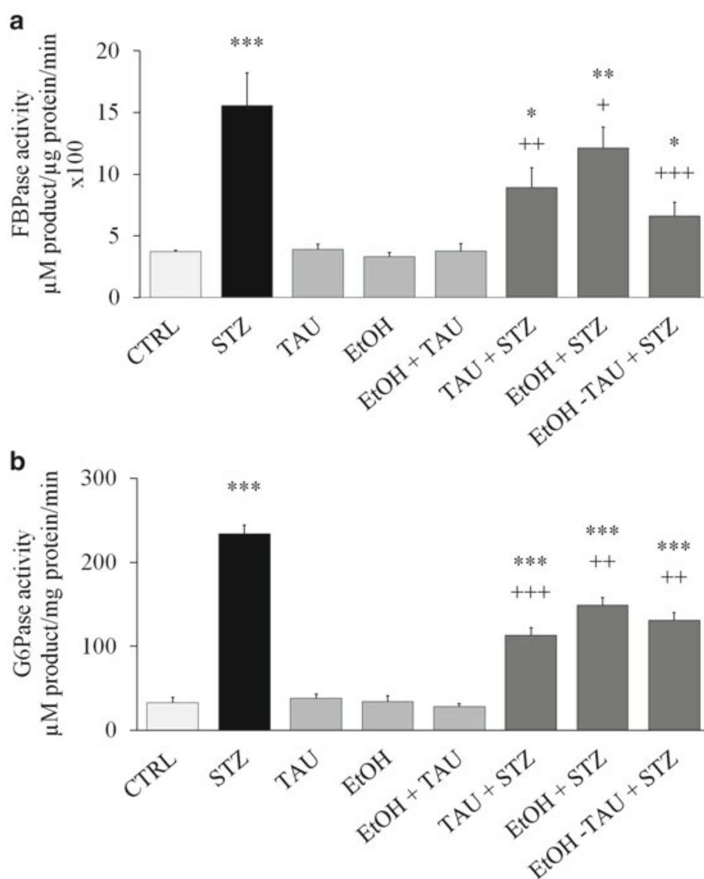

Fig. 6 Renal activities of (**a**) FBPase and (**b**) G6Pase of diabetic (STZ) rats in the absence and presence of 5 % EtOH, TAU or 5 % EtOH plus TAU. Values are shown as mean ± SEM for $n = 6$. Differences were statistically significant vs. control at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$; and vs. STZ at $^{+}p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$

respectively, both at $p < 0.01$ vs. diabetes), an effect that became enhanced in the presence of both agents (only 17 % increase, $p < 0.05$ vs. control) (Fig. 7a, b). Neither EtOH nor TAU exerted a significant altering effect on the cMDH activities of naive rats.

3.5 *Enzymes of the Pentose Phosphate Pathway*

An estimate of glucose flux through the renal pentose phosphate pathway was obtained from the measurement of the activities of G6PDH (Fig. 8a) and 6PGDH (Fig. 8b), which were found to be substantially higher in diabetic than in control rats (by 200 % and 239 %, respectively, $p < 0.001$, respectively). Both EtOH and TAU

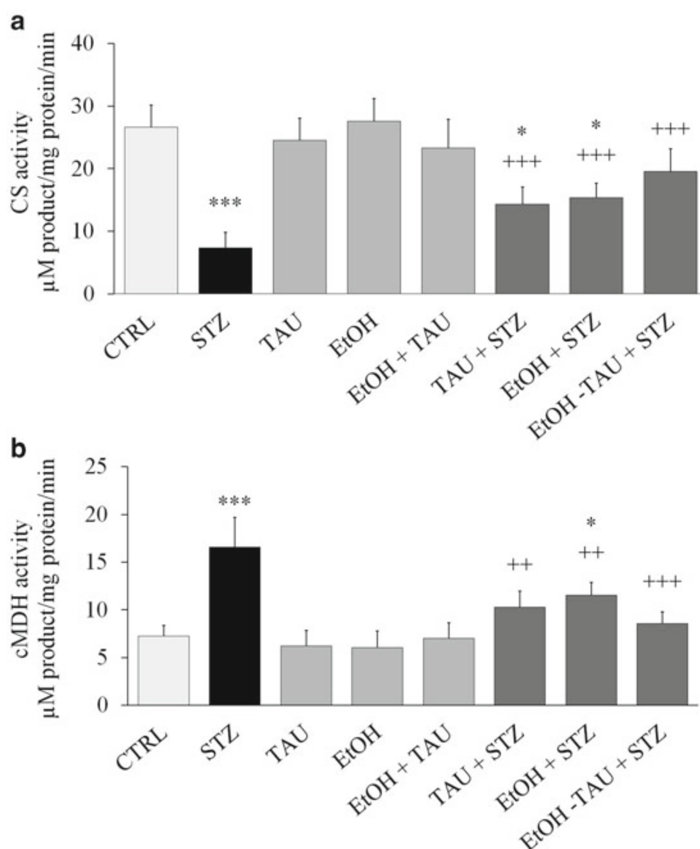

Fig. 7 Renal activities of (**a**) CS and (**b**) cMDH of diabetic (STZ) rats in the absence and presence of 5 % EtOH, TAU or 5 % EtOH plus TAU. Values are shown as mean ± SEM for $n=6$. Differences were statistically significant vs. control at $^{*}p<0.05$ and $^{***}p<0.001$; and vs. STZ at $^{++}p<0.01$ and $^{+++}p<0.001$

effectively reduced these increases, with a combined treatment providing a greater reduction (increases of only 38 %, $p<0.01$, and 39 %, $p<0.01$, respectively) than the individual treatments (only 89 % and 54 %, respectively, in the case of G6PDH; only 89 % and 94 % increase in the case of 6PGDH, all at $p<0.001$ vs. control values).

3.6 Enzymes of the Sorbitol Pathway

The polyol pathway entails the activity of two enzymes, AR, found to reduce glucose to sorbitol in the presence of its cofactor NADPH, and SDH, which requires NAD^+ as a cofactor to converts sorbitol to fructose. In the present study, diabetes affected these enzymes quite differently, with the activity of AR rising (by 109 %, $p<0.001$) and that of SDH showing a negligible decrease (10 %) decrease relative to the values

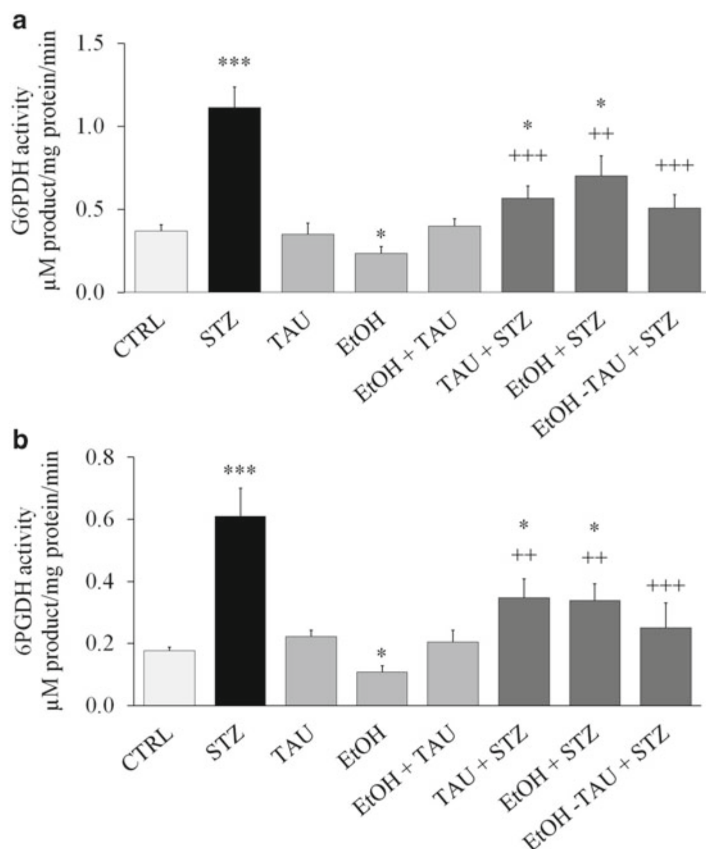

Fig. 8 Renal activities of (**a**) G6PDH and (**b**) 6PGDH of diabetic (STZ) rats in the absence and presence of 5 % EtOH, TAU or 5 % EtOH plus TAU. Values are shown as mean ± SEM for $n = 6$. Differences were statistically significant vs. control at $^{*}p < 0.05$ and $^{***}p < 0.001$; and vs. STZ at $^{++}p < 0.01$ and $^{+++}p < 0.001$

for normal rats (Fig. 9a, b, respectively). By itself, EtOH reduced the loss of AR (by 36 %, $p < 0.01$ vs. diabetes) but was unable to normalize the diabetic SDH activity. A daily treatment with TAU was insignificantly less effective than EtOH on the SDH activity (29 % decrease, $p < 0.01$ vs. diabetes) and as ineffective as EtOH on the diabetic SDH activity (Fig. 10). When available individually or together, neither EtOH nor TAU had an appreciable effect on the activity of polyol pathway in naive rats.

3.7 Kidney Glucose and Glycogen Levels

As shown in Figs. 10a, b, both glucose and glycogen were markedly elevated in the kidney of diabetic rats (by 179 % and 232 %, respectively) when compared with the corresponding control values (both at $p < 0.001$) The same figures show the effects

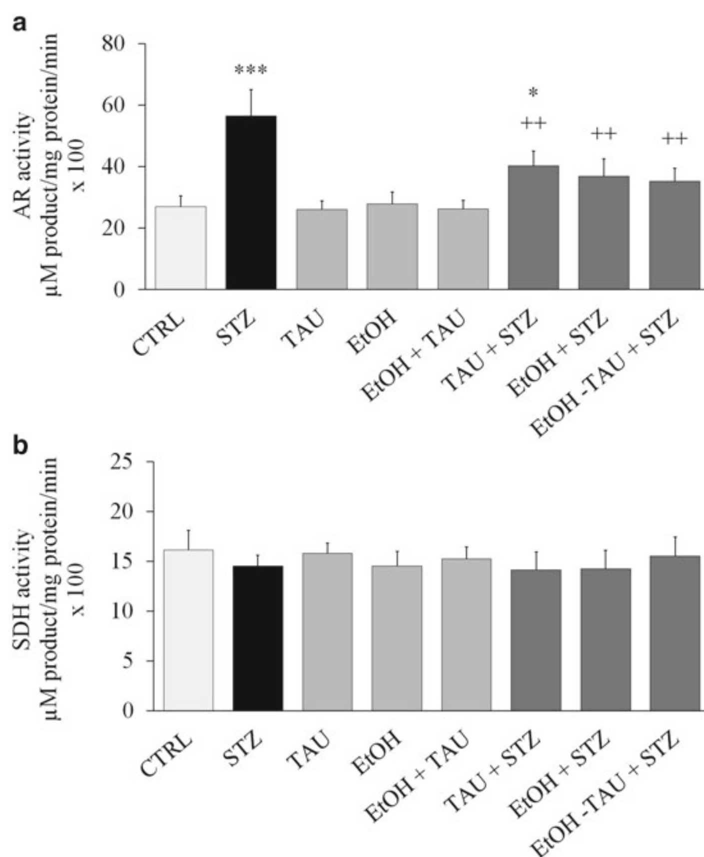

Fig. 9 Renal activities of (**a**) AR and (**b**) SDH of diabetic (STZ) rats in the absence and presence of 5 % EtOH, TAU or 5 % EtOH plus TAU. Values are shown as mean ± SEM for $n=6$. Differences were statistically significant vs. control at $^{*}p<0.05$ and $^{***}p<0.001$; and vs. STZ at $^{++}p<0.01$

of EtOH and TAU, alone and in combination, on these renal enzyme activity changes. The daily intake of EtOH, starting before the onset of diabetes resulted in a significant reduction of these increases (glycogen by 21 % and glucose by 26 %, respectively, both at $p<0.05$ vs. diabetes). Using the same treatment approach, a daily oral treatment with TAU was without effect on the diabetic glucose level but it caused a significant lowering effect on the accumulation of renal glycogen (38 % less, $p<0.01$). Combining the intake of EtOH with a TAU treatment exerted a greater lowering effect on the diabetic levels of glucose (72 % less) and glycogen (50 % less) than when these treatments were provided individually (both at $p<0.001$ vs. diabetes). In naive rats EtOH and TAU were found to exert only a mild enhancing effect of the renal glucose and a mild lowering effect on the renal glycogen. The aforementioned changes in renal glycogen and glucose were also reflected on the corresponding glycogen/glucose ratios. As shown in Fig. 10c, in the diabetic kidney this ratio rose dramatically in comparison to the control value. Regardless of

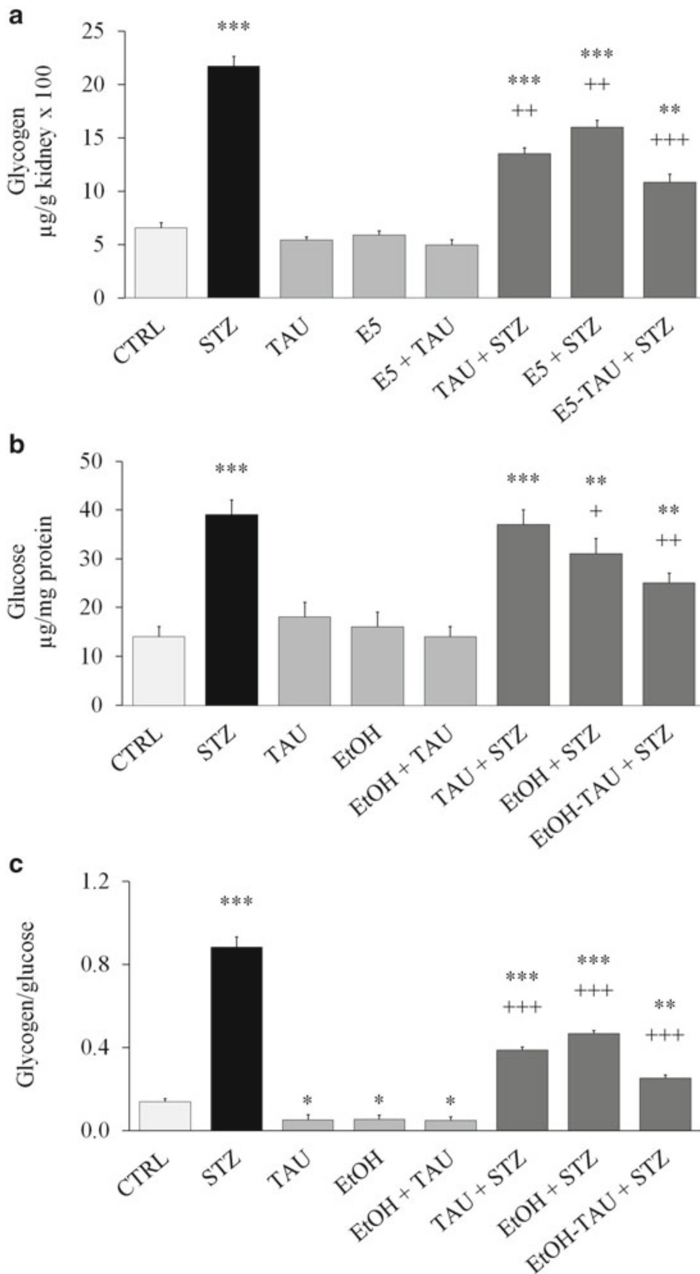

Fig. 10 Renal values for (**a**) glycogen, (**b**) glucose and (**c**) glycogen/glucose ratio of diabetic (STZ) rats in the absence and presence of 5 % EtOH, TAU or 5 % EtOH plus TAU. Values are shown as mean ± SEM for $n = 6$. Differences were statistically significant vs. control at $^{**}p < 0.01$ and $^{***}p < 0.001$; and vs. STZ at $^{+}p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$

whether they were available singly or as a combination, EtOH and TAU were found to reduce the ratio increase by more than one-half of the diabetic value, more so when available as a combination.

4 Discussion

4.1 Diabetes, Plasma Glucose and Renal Activities of Glycolytic and Gluconeogenic Enzymes

A chemically-induced animal model of diabetes was used to examine the effects of diabetes on classical and alternative pathways of glucose metabolism in the kidney, and to evaluate the effect of a low concentration (5 %) of EtOH and TAU, provided singly and in combination, on the activities of these pathways. The dose of STZ used to induce diabetes is one previously shown in this laboratory to cause a marked (>300 mg/dL) elevation of the plasma glucose and to maintain a subnormal capacity to secrete insulin (Pandya et al. 2013). The uninterrupted oral availability of EtOH or TAU before and after the induction of diabetes with STZ, led to a lowering of the diabetic plasma glucose to a level that was markedly different from that of untreated diabetic rats. Studies with perfused rat liver and incubated kidney cortex slices have revealed EtOH to sharply curtail gluconeogenesis in the liver but not in the kidney, and from lactate but not from pyruvate, probably due to the accumulation of NADH arising from hepatic EtOH metabolism (Krebs et al. 1969). The same explanation could account for the decreased utilization of glucogenic amino acids such as glutamate and of amino acids capable of entering the TCA cycle (Frieden 1959), although the possibility that EtOH could induce hypoglycemia by interfering with the oxidative deamination of glucogenic amino acids, as inferred from the decrease in urea production from amino acid nitrogen, has also been considered (Field et al. 1963). In spite of these lines of evidence on the role of EtOH on diabetic gluconeogenesis, an alternative explanation is required since EtOH did not alter the baseline plasma glucose level by more than 15 %. A similar situation surrounds the hypoglycemic action of TAU, which has been found in some (Ersöz et al. 1994; Nakaya et al. 2000), but not all (Goodman and Shihabi 1990; Tappia et al. 2013), studies to be able to lower the circulating levels of glucose in animal models of diabetes.

In agreement with the findings of earlier studies in the liver of diabetic patients and rats (Wilms et al. 1970), the activities of the glycolytic enzymes HK-1, GKL, PFK-1, PK were higher in the kidney of STZ-treated than of control rats, except for that of GAPDH which was lower. Most studies have found the activities of glycolytic enzymes to increase in the jejunal mucosa (Anderson 1974), and skeletal muscle and renal cortex of rats with mild to moderate diabetes (Anderson and Stowring 1973; Murphy and Anderson 1972), but there is at least one study reporting the opposite effects on the activities of the rate-limiting enzymes HK, PFK-1 and PK in the liver of STZ-treated rats (Anderson and Stowring 1973). In contrast, the activity

of GAPDH has been consistently found decreased in erythrocytes of diabetic patients (Fleming et al. 2012), in cultured RAW1264.7 cells (Easterday et al. 2007), and in the retina of STZ-treated diabetic mice (Kanwar and Kowluru 2009). This inhibition may stem, at least in part, to the presence of a highly reactive thiol group at its catalytic domain that makes this enzyme highly susceptible to modification by free radicals (Hyslop et al. 1988; Kanwar and Kowluru 2009), peroxynitrite and other nitrosating agents (Mohr et al. 1994), and glycating agents (Beisswenger et al. 2003; Easterday et al. 2007; He et al. 1995) with the subsequent loss of its activity. In addition, a noncompetitive inhibition by endogenous aldehydes (Novotny et al. 1994) and a limited availability of NAD^+ for GAPDH activity as a result of increased utilization during the conversion of glucose to sorbitol by SDH in the polyol pathway (Taylor and Agius 1988) have also been proposed in support of the inhibition.

The continuous intake of 5 % EtOH, before and after the induction of diabetes with STZ, resulted in a significant lowering of the activities of all the enzymes tested, particularly that of GAPDH, which is recognized as the main target for the inhibitory action of EtOH on glycolysis. In this case, EtOH is limiting the availability of NAD^+ for the activity of GAPDH by decreasing the NAD^+/NADH ratio as a result of its cytosolic oxidation by alcohol dehydrogenase (ADH) (Badawy 1977). In this regard, TAU was more effective than EtOH in lowering the activity change induced by diabetes, especially when used in conjunction with EtOH. TAU is known to exert a positive effect on glycolysis by enhancing flux through PFK-1 and by potentiating the action of insulin (Lampson et al. 1983). Individually or together, none of these treatment agents affected the control enzyme values to a significant extent.

Along with the liver, the kidney represents an important source of the glucose released into the circulation in the postabsorptive state (Gerich et al. 2001) and during periods of prolonged fasting (Ekberg et al. 1999; Gerich et al. 2001) to maintain euglycemia. To meet this role, the kidney is equipped with gluconeogenic enzymes which, in terms of organ weight, are in a higher concentration than in the liver in spite of displaying comparable blood flows. In common with the results of studies on the liver of type 2 diabetic patients (Magnusson et al. 1992) and isolated proximal tubules from fatty Zucker diabetic rats with the characteristics of type 2 diabetes (Eid et al. 2006), the present study found the activities of the gluconeogenic enzymes cPEPCK, G6Pase and FBPase to be higher in the kidneys of diabetic than of control rats. Studies in humans and in animal models of type 2 diabetes have demonstrated that the activities of these enzymes are largely confined to the renal cortex (Anderson and Stowring 1973; Stumvoll et al. 1997), with the renal proximal tubule representing an additional gluconeogenic site (Eid et al. 2006; Guder and Ross 1984). In contrast to the liver, the ingestion of glucose can increase both renal gluconeogenesis and glucose uptake into the kidney (Mather and Pollock 2011). Increases in glucose flux through both glycolysis and gluconeogenesis have also been verified during the hyperglycemia of long-term clinical (Mitrakou 2011) and experimental (Nannipieri et al. 2001) diabetes. Both 5 % EtOH and TAU lowered the diabetic activities of gluconeogenic enzymes, with TAU providing a greater effect than 5 % EtOH. The attenuation was enhanced when both treatments were available concurrently, possibly as a result of independent mechanisms of action.

4.2 Diabetes and Enzyme Activities of the TCA Cycle, cMDH and Pentose Phosphate Pathway

In comparison to control rats, diabetic ones demonstrated a much lower activity of CS serving as an indicator of the activity of the TCA cycle. This result is in agreement with that of a study carried out in rats made diabetic with alloxan and which demonstrated a marked decreased in the concentration of TCA cycle intermediates in the liver and kidney (Frohman et al. 1951). The marked decreases in the activities of HK and CS and in the decarboxylation of 1-^{14}C and 2-^{14}C pyruvate by lymphocytes from alloxan-treated rats was taken as indication that diabetes affect the flux of metabolites through glycolysis, the pyruvate dehydrogenase complex and the TCA (Otton et al. 2002). A decrease in insulin-mediated CS activity has been verified in cultured skeletal muscle cells from patients with type 2 diabetes and exposed to insulin and/or palmitate. Since there were no changes in mitochondrial respiration or in 3-hydroxyacyl-CoA dehydrogenase activity yet a correlation existed between the activity of CS and the rate of synthesis of fatty acids, it was concluded that the insulin resistance at the CS level seen in diabetes is due to an exposure to high levels of free fatty acids (Ortenbland et al. 2005). Moreover, a significant increase in citrate concentration has been observed in the kidney, liver and pancreas of alloxan-treated mice, an effect that has been ascribed to a concurrent inhibition of mitochondrial aconitase, the enzyme immediately next to CS in the TCA cycle (Boquist et al. 1985).

The oxidation of EtOH by the liver and other tissues possessing ADH activity increases the supply of reducing equivalents in the form of NADH which, after transport into the mitochondria, can inhibit the TCA cycle at the CS and isocitrate dehydrogenase steps (Williamson et al. 1969). When provided singly, before and after the induction of diabetes, both EtOH and TAU were found to increase the diabetic CS activity twofold. In addition, the simultaneous availability of EtOH and TAU demonstrated a greater elevating effect on the diabetic CS activity than either treatment alone. Since neither EtOH nor TAU altered the baseline value of CS to a significant extent, it is safe to assume that at their protecting by influencing a diabetes-mediated change such as that of the ratios of pyridine nucleotides (as NAD^+/NADH) and of ATP/ADP (Obrosova and Stevens 1999; Williamson et al. 1969).

The cMDH is an isoform of MDH that represents the rate-limiting enzyme for the malate-aspartate shuttle, which is involved in glucose metabolism and in the transport of cytosolic reducing equivalents, originating from glycolysis as NADH, across the mitochondrial membranes for utilization by the electron transport chain. Most of the work on the effects of diabetes on MDH has been focused on the hepatic mitochondrial isoform activity, which has been found to increase or decrease in response to the diabetes induced by different chemical diabetogens (Panneerselvam and Govindaswamy 2002; Popov et al. 1998; Ramudu et al. 2011), with only a limited attention having been devoted to the cytosolic isoform. In contrast to the results derived for the cytosolic MDH isoform from diabetic dogs and cats, indicating that

both the MDH activity and MDH RNA expression are decreased (Magori et al. 2005), in the present study the renal activity of this enzyme was much higher in diabetic than in normal rats. A similar, although less marked, increase has been observed in the adipose tissue of diabetics patients relative to normal patients (Belfiore et al. 1975). The various treatment approaches were able to significantly lower the increase in renal cMDH activity ensuing diabetes, with TAU providing the least protection and TAU plus EtOH the greatest protection was effectively attenuated by EtOH, to a lesser extent by TAU, and to a greater extent by a combined pretreatment with both agents. None of these treatment approaches exerted an appreciable effect on the cMDH activities of naive rats.

An estimate of the renal flux of glucose through the pentose phosphate pathway was obtained by measuring the activities of G6PDH and 6PGDH, two enzymes of the oxidative segment of the pathway. In the present study, the activities of both enzymes were significantly higher (by 3- and 3.4-fold, respectively) than corresponding control values, with the magnitude of the increases comparing favorably with those reported in the literature for adults STZ-treated rats (Sochor et al. 1986). Chronologically speaking, it appears that the activities of these enzymes in this animal model of diabetes are maximally increased during the first week of the induction of diabetes, with the activity declining towards control values thereafter (Steer et al. 1985). Based on the effects of EtOH on G6PDH, it is reported that EtOH lowers the activity of the pentose phosphate pathway in the liver and enhances it in the brain and skeletal muscle (Badawy 1977; Lelevich 2008). In this work a 5 % EtOH solution was found to have little effect on the baseline activity value of G6PDH and 6PGDH in the kidney, even in the presence of TAU. The increases in renal activities of these two enzymes by diabetes could represent a response to the oxidative stress of diabetes since the pentose pathway is a source of reducing power for the main cellular redox systems (Grant 2008) and neutralization of prooxidants (Palmer 1999). The greater reduction in enzyme activity achieved in the presence of TAU, a known antioxidant, than of EtOH alone lends support to this concept. In naive rats, 5 % EtOH, but not TAU or TAU plus 5 % EtOH, was found to exert a significant lowering effect on the activities of G6PDH and 6PGDH. It is possible that EtOH is directly inhibiting the activity of these enzymes rather than through the increase in the $NADP^+$/NADPH ratio demonstrate in the liver under acute and chronic conditions of EtOH exposure (Badawy 1977).

4.3 Diabetes and the Polyol Pathway

The kidney differs from the liver and adipose tissue in that it does not require insulin for glucose transport and phosphorylation (Sochor et al. 1988). As a result, in diabetes the entry of glucose into the kidney is increased and, upon subsequent metabolism by the polyol pathway enzymes AR and SDH, it contributes to the intracellular accumulation of sorbitol and fructose, respectively (Sochor et al. 1988). In

agreement with the results of work by Corder et al. (1979) with glomeruli from kidneys of human diabetics and with lenses of rats receiving STZ (Varma and Kinoshita 1974), the present study determined a significant increase in AR activity together with a negligible decrease in SDH activity in the diabetic rat kidney. These enzymatic changes, along with an increase in cytosolic $NADP^+$/NADPH and decrease in NAD^+/NADH ratios (Obrosova and Stevens 1999), will favor sorbitol accumulation and, thus, contribute to the pathogenesis and progression of glomerulosclerosis and other microvascular complications of diabetes.

In the present study, the intake of 5 % EtOH by diabetic rats led to a significant reduction in AR activity. This effect has been related to the metabolism of EtOH by the cytosolic NAD^+-dependent ADH of the liver and kidney, and which will led to an increase in NADH to level sufficiently high to competitively inhibit SDH at low levels of NAD^+*in vitro* (Hillbom 1970; Hillbom and Pikkarainen 1970) but possibly not *in vivo* (Hillbom and Lindros 1971). In contrast, EtOH exerted an insignificant effect on the activity of SDH, possibly because of the already low value seen with diabetes. In comparison to EtOH, TAU demonstrated an insignificantly weaker reducing effect on the diabetic ADH activity and an equivalent effect on the diabetic SDH activity. On the other hand, a combined treatment with EtOH plus TAU resulted in an attenuation of a similar extent to that achieved with EtOH alone. The protective actions of TAU may be related to its ability to ameliorate the decrease in cytosolic NAD^+/NADH ratio, ATP/ADP ratio and adenylate energy charge (Obrosova and Stevens 1999). Both EtOH and TAU were without effect on the ADH and SDH activities of naive rats.

4.4 Diabetes and Renal Glucose and Glycogen

In agreement with the results of studies conducted in untreated diabetic patients and in animal models of chemically-induced diabetes, this study verified both glucose and glycogen to be higher in the kidney of diabetic than of normal rats. Histological evaluation of kidney sections has determined a pronounced accumulation of glycogen in the cytoplasm of renal tubular epithelial cells (Holck and Rasch 1993; Nannipieri et al. 2001) that was directly proportional to the increase in blood glucose concentration (Tsuchitani et al. 1990). Although the kidney glycogen content in rats with STZ-induced diabetes has been consistently found increased, that has not been the case as it relates to the activities of renal glycogen-metabolizing enzymes. For example, an increase in kidney glycogen phosphorylase was found accompanied by a corresponding decreased in that of glycogen synthase (Nannipieri et al. 2001). However, another study found the activities of glycogen phosphorylase, phosphorylase kinase, and protein kinase not to be significantly altered, the activity of glycogen synthase I to be decreased, and the total glycogen synthase (I + D) activity to increase in unison with the levels of glucose 6-phosphate, an activator of glycogen synthase (Khandelwal et al. 1979). In the face of such discrepancies, it has been suggested that glycogen-related enzymatic changes are secondary to glycogen

accumulation and that hyperglycemia is the sole driving force for this phenomenon (Nannipieri et al. 2001).

The present study found that the daily intake of a low concentration EtOH solution by diabetic rats raised the kidney glycogen content to a level that more than twofold above the control value but which did not reach the levels seen in diabetic rats not exposed to EtOH. The increase in renal glycogen seen in diabetic rats may reflect the greater influx of circulating glucose into the kidney (Mather and Pollock 2011), activation of glycogenesis, inhibition of glycogenolysis or the regulation of both processes (Khandelwal et al. 1979), while the decrease in diabetic glycogen in the presence of low EtOH could have resulted from the lower plasma and renal glucose levels that follow the improvement in extrarenal glucose utilization due to the increase in insulin sensitivity fostered by low to moderate EtOH consumption (Feng et al. 2008; Furuya et al. 2005). The present results dramatically contrast not only with the effects of low EtOH in the diabetic rat liver where the glycogen content is much lower than in control rats (Khandelwal et al. 1979) but also with the extensive depletion of glycogen caused by heavy EtOH consumption (Emanuele et al. 1998; Martin et al. 2004; Van Horn et al. 2001) or treatment with high doses of EtOH (Kubota et al. 1992), which are known to stimulate hepatic glycogenolysis while reducing glycogenesis, gluconeogenesis and glucose transporter concentration in the liver (Van Horn et al. 2001), and to induce insulin resistance (Ohnishi et al. 2003). At a 5 % concentration EtOH was devoid of a significant effect on both the renal glycogen and glucose contents.

From the limited information on the effects of TAU on glycogen metabolism and on the intracellular concentrations of glycogen in tissues with a high gluconeogenic capacity such as the liver and kidney, it appears that this amino acid can increase the cellular uptake of glucose accumulation in an insulin-like manner but without enhancing insulin release and in a tissue-specific manner (Kulakowski and Maturo 1984; Maturo and Kulakowski 1988), and to stimulate glycogen synthesis in adipocytes, the liver and heart (Maturo and Kulakowski 1988). The present results confirm this stimulatory effect but which was weaker than that of EtOH and much weaker than that of diabetes. However, when it was concurrently available with EtOH, it reduced the renal glycogen level to a value that was lower than with each agent alone. This effect might be related to the suppression of STZ-induced hyperglycemia stemming from the protection conferred by TAU against pancreatic β-cell damage by STZ (Tokunaga et al. 1979).

5 Conclusion

This study finds that the metabolism of glucose by classical and alternative pathways is variously affected by diabetes and that 5 % EtOH and/or TAU can attenuated these alterations. On the basis of the activities of key metabolic enzymes, diabetes is found to stimulate glycolysis, gluconeogenesis, polyol pathway, pentose phosphate pathway and malate shuttle, to inhibit the Krebs cycle, and to favor a greater renal

accumulation of glycogen over that of glucose. Both 5 % EtOH and TAU are able to attenuate these alterations and the underlying hyperglycemia to a significant and about equal extent when made available on a daily basis. Moreover, an enhanced attenuating effect was achievable through a concurrent treatment with these agents.

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Hypoglycemia Is One Possible Mechanism in the Combined Toxicity of Ethanol and Taurine

Andrey G. Taranukhin, Pirjo Saransaari, Kalervo Kiianmaa, and Simo S. Oja

1 Introduction

Taurine (2-aminoethanesulfonic acid) is a simple sulfur-containing amino acid which is ubiquitously distributed in the tissues of most animals, including mammals (Oja and Kontro 1983). For instance, a human weighing 70 kg contains up to 70 g of taurine (Huxtable 1992). Taurine is not incorporated into tissue proteins and is thus present as a free amino acid. It occurs at higher concentrations than other amino acids in tissues such as muscle, platelets, retina and the central nervous system (Lourenço and Camilo 2002). Taurine is involved in a wide range of physiological processes including osmoregulation, lipid metabolism, intracellular calcium regulation, neuronal development, neuromodulation and cell protection (Huxtable 1992; Oja and Saransaari 1996; Ripps and Shen 2012).

Taurine has become a common component in alcoholic and nonalcoholic energy drinks (Ayala et al. 2009; Lutmer et al. 2009; Higgins et al. 2010). Studies on energy drinks and human health might offer a good source of knowledge as to the combined effects of taurine with alcohol and other drink components. However, the studies available on the possible adverse effects of energy drinks have focused mainly on caffeine overdoses (Reissig et al. 2009; Seifert et al. 2011; Gunja and Brown 2012; Rath 2012; Wolk et al. 2012) and on the adverse effects of the combination of caffeine and alcohol (Weldy 2010; Wolk et al. 2012). They provide no evidence as to the possible toxicity of taurine alone or its combination with alcohol (Rath 2012). Our recent findings (Taranukhin et al. 2013) are thus the first indication of the combined toxicity of taurine and ethanol, emphasizing the potential

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danger of energy drinks for humans. Measurements of blood glucose in adult and old mice after co-administration of high doses of taurine and ethanol suggested that one possible mechanism of toxicity might be hypoglycemia. In the present study we focused mainly on effects of taurine and ethanol co-administration on blood glucose in 7-day-old mice and compared these results with data obtained from adult and elderly mice.

2 Methods

2.1 Animals and Experimental Procedure

Pregnant 8-week-old NMRI female mice were purchased from Harlan (Horst, the Netherlands). In our experiments we used 7-day-old pups (males and females). Day of birth is day 0 (zero). The mice were divided into five experimental groups—intact, control, ethanol-treated, taurine-treated and ethanol + taurine-treated. Ethanol (20 % w/v solution diluted in saline) and taurine (7 % w/v solution diluted in saline) were administered subcutaneously to 7-day-old mice. The taurine and ethanol + taurine groups were divided into three sub-groups according to the different taurine doses provided (2 g/kg, 4 g/kg and 6 g/kg). Ethanol was applied in a total dose of 5 g/kg to ethanol-treated and ethanol + taurine-treated groups. Taurine and ethanol were injected in two half-doses: taurine at 0 h and 4 h, ethanol at 1 h and 3 h. The control animals were given saline injections equal to those in the ethanol + taurine-treated group. Thirty minutes after the last injection the animals were decapitated and blood used for glucose level determination.

2.2 Blood Glucose Measurement

By reason of the small size of the 7-day-old mice (4.5–6.0 g) we were able to take only one blood sample from each animal. Samples of 5 μl blood were collected into HemoCue Glucose cuvettes and immediately analyzed in a HemoCue B-Glucose Analyzer (HemoCue AB, Ängelholm, Sweden). Intact mice (at least one male and one female from each litter) were used to establish a baseline for each litter, where other pups were used in experimental groups.

2.3 Statistic Analysis

Statistical significance was determined by Student's *t*-test. Each value is expressed as the mean ± SD. Differences were considered statistically significant when the calculated p value was less than 0.05.

3 Results

3.1 Changes in the Blood Glucose Level After Taurine and Ethanol Co-administration in 7-Day-Old Mice

The 7-day-old mice treated with 6 g/kg of taurine combined with 5 g/kg of ethanol usually died within 30–60 min after the last taurine injection. We measured glucose in blood 30 min after the last taurine injection to detect the blood glucose maximum close to animal death. Saline injections to the control group did not change glucose level in blood if compared with intact mice. Ethanol applied at a total dose of 5 g/kg likewise did not alter the blood glucose level when compared to the intact and control groups. Taurine administration significantly reduced blood glucose compared to both intact and control mice. It seems that the drop in glucose appeared in a dose-dependent manner (Fig. 1). Co-administration of ethanol and taurine also significantly lowered blood glucose in comparison to intact and control mice. The decrease in blood glucose after ethanol and taurine co-administration appeared to be even greater than in mice treated with taurine only, but the difference was significant only in the ethanol + taurine 6 g/kg and taurine 6 g/kg groups. The decrease in blood glucose after ethanol and taurine co-administration depended on the taurine dosage.

4 Discussion

The beneficial effects of taurine have been shown in many pathological conditions such as ischemia (Takatani et al. 2004; Taranukhin et al. 2008), high glucose level (Ulrich-Merzenich et al. 2007) and oxidative stress (Das et al. 2009). Previously, we have shown the neuroprotective effect of taurine against ethanol-induced apoptosis. Taurine at a dose of 2 g/kg applied to ethanol-treated 7-day-old mice saved about 50 % of dying neurons (Taranukhin et al. 2009, 2010, 2012). However, when we increased the taurine doses to enhance the beneficial effects, we obtained the unexpected result that high doses of taurine co-administered with ethanol kills the treated animals (Taranukhin et al. 2013). Since the high doses of taurine alone or ethanol alone did not lead to animal death, lethality appears to be due to ethanol and taurine combined toxicity. We also found that the toxicity of ethanol and taurine combination is age-dependent. The 100 % lethal doses of ethanol and taurine co-administration for different age groups are presented in Table 1.

The lethal doses in ethanol and taurine co-administration resulted in a significant drop in blood glucose immediately prior to death, being in adult (5–6-month-old) 40 % less and in old (12–13-month-old) mice 50 % less than in the same animals before treatments. This finding allowed us to assume that the hypoglycemia may be one of possible reason for ethanol and taurine combined toxicity (Taranukhin et al. 2013). The ability of taurine to reduce blood glucose has already been demonstrated

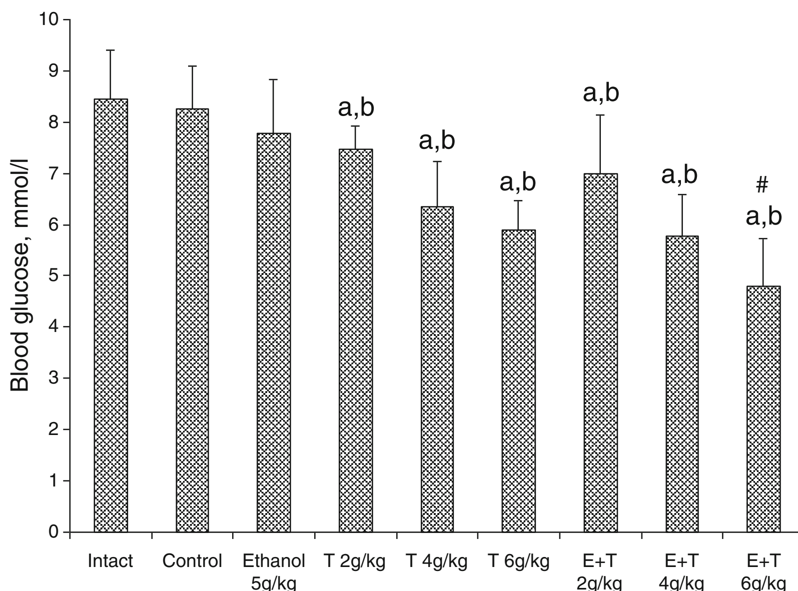

Fig. 1 Decrease in blood glucose in 7-day-old mice after taurine and ethanol co-administration. Intact, $n=19$; control, $n=10$; ethanol 5 g/kg, $n=10$; T 2 g/kg, $n=5$; T 4 g/kg, $n=5$; T 6 g/kg, $n=11$; E+T 2 g/kg, $n=10$; E+T 4 g/kg, $n=10$; E+T 6 g/kg, $n=16$. The significance of differences compared to the intact group: "a" $p<0.01$. The significance of differences compared to the control group: "b" $p<0.05$. The significance of differences between the T 6 g/kg and E+T 6 g/kg groups: # $p<0.001$. Abbreviations: *E* ethanol, *T* taurine

Table 1 Taurine and ethanol combined toxicity

Age groups	100 % Lethal doses
7-day-old	Ethanol 5 g/kg + taurine 6/kg
5–6-month-old	Ethanol 8 g/kg + taurine 10 g/kg
12–13-month-old	>Ethanol 6 g/kg + taurine 6 g/kg

in rats (Nakagawa and Kuriyama 1975; Kulakowski and Maturo 1984), rabbits (Winiarska et al. 2009) and guinea pigs (Kaplan et al. 2004), indicating the potential beneficial effects of taurine in the therapy of diabetes. In our experiments on 7-day-old mice taurine alone significantly reduced blood glucose in a dose-dependent manner (Fig. 1). Previously, we obtained similar results with old mice, where taurine alone likewise markedly lowered blood glucose. In adult mice, however, the blood glucose level did not change after taurine treatment (Taranukhin et al. 2013).

It is known that alcohol ingestion may cause hypoglycemia (Truman and Picchi 1965; Yang et al. 1995; Huang and Sjöholm 2008). However, in our experiments acute ethanol treatment did not change blood glucose in 7-day-old (Fig. 1) or adult and old mice (Taranukhin et al. 2013).

Co-administration of taurine and ethanol induced a significant decrease in blood glucose in 7-day-old mice. The degree of this effect depended on the taurine dosage

applied. It seems that with an equal taurine dose the decrease in glucose is greater in the group where animals were also treated with ethanol, but this difference was statistically significant only with the highest taurine dose (Fig. 1). In adult and old mice the fall in blood glucose was also markedly greater in ethanol + taurine-treated mice than in the taurine-treated group (Taranukhin et al. 2013). Comparison of the blood glucose drop after ethanol and taurine co-administration between different age groups shows that this treatment lowers glucose in blood to about 40–50 %—from 8–10 mmol/l in the control groups to 4–6 mmol/l in the ethanol + taurine-treated groups (Fig. 1; Taranukhin et al. 2013). The blood glucose level equal to 4–6 mmol/l is far from the fatally dangerous hypoglycemic level and could not be the reason for death induced by taurine and ethanol combined toxicity. However, there was wide variation between individual animals in blood glucose after taurine and ethanol co-administration. For example, in old mice the blood glucose level varied from 6.0 mmol/l in some animals to 2.2 mmol/l in others. Such markedly low blood glucose (2.2 mmol/l) may be a reason for death (Taranukhin et al. 2013). The heterogeneity in blood glucose levels in 7-day-old, adult and old mice is presented in Fig. 2. In 7-day-old mice the animals after treatment with a 100 % lethal combination of taurine and ethanol were divided into two sub-groups with 5.23 ± 0.57 mmol/l (75 % of animals) and 3.5 ± 0.27 mmol/l (25 % of animals). The difference between these subgroups was statistically significant (Fig. 2). Adults from the ethanol + taurine-treated group were not divided into sub-groups, in view of the small variation within the group and the small number of mice in the group. Old mice from the ethanol + taurine group were divided into two sub-groups with

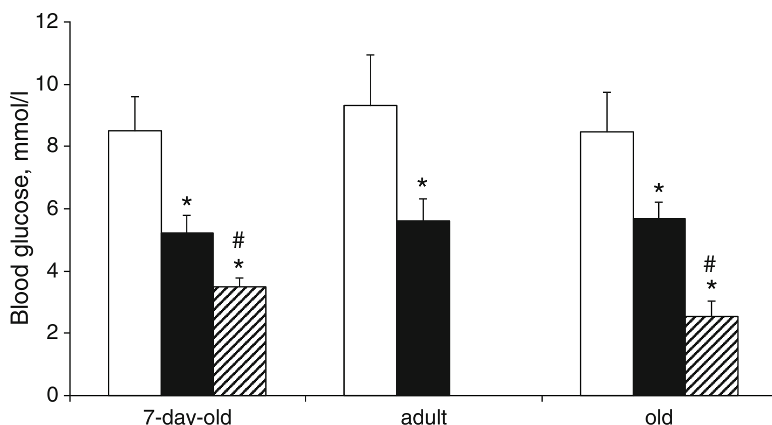

Fig. 2 Heterogeneity in blood glucose after taurine and ethanol co-administration. Intact (*open bars*), two sub-groups of ethanol + taurine-treated mice (higher glucose level—*filled bars*; lower glucose level—*striped bars*). 7-day-old mice: open bar, $n = 9$; filled bar, $n = 12$; striped bar, $n = 4$. Adult mice (5–6-month-old): open bar, $n = 3$; filled bar, $n = 3$. Old mice (12–13-month-old): open bar, $n = 7$; filled bar, $n = 3$; striped bar, $n = 2$. The significance of differences compared to open bar: *$p < 0.05$. The significance of differences between the *filled* and *striped bars*: #$p < 0.05$

5.7 ± 0.52 mmol/l (60 % of animals) and 2.55 ± 0.49 mmol/l (40 % of animals). Interestingly, after treatment with 100 % of lethal doses of combined ethanol and taurine 100 % of animals died in all age groups. However, only 25–40 % of the animals which died had a low hypoglycemic level of blood glucose (2.2–3.5 mmol/l), which can be considered the reason of death. It seems that another mechanism or mechanisms of combined ethanol and taurine toxicity must exist.

Although the study of the mechanisms of death caused by hypoglycemia is beyond the scope of this study, we believe it is appropriate to consider them shortly now as the results of our present work show a strong hypoglycemic effect of taurine and ethanol combination. It is known, that hypoglycemia commonly causes brain fuel deprivation, resulting in functional brain failure, which can be corrected by increasing plasma glucose concentrations. Rarely, profound and prolonged hypoglycemia causes brain death. In humans, physiological normal range of blood glucose is about 3.9–6.1 mmol/l. A decrease in blood glucose to 3.8 mmol/l increases the glucagon and epinephrine secretions aimed to restore the normal blood glucose level. If by any reason the blood glucose concentration continues to fall, functional brain failure ensues which manifests itself as cognitive impairment (2.8 mmol/l), aberrant behavior, seizures and coma (2.3–2.7 mmol/l). A further decrease in blood glucose to 1 mmol/l or lower induces neuronal death and is lethal (Cryer 2007). As reported by Tanenberg et al. (2010) the fall in blood glucose to 1.5 mmol/l could be considered a sole cause of death of a 23-years-old diabetic patient. However, brain death is not only a single cause of death induced by hypoglycemia. During hypoglycemia the heart beat rate and systolic blood pressure are elevated, which increase coronary blood flow and oxygen consumption. These effects may result in arrhythmia, ischemia, and even death (Lalic 2013). Above we have described harmful effects of profound hypoglycemia. However, mild hypoglycemia (2.2–3.5 mmol/l) may also be dangerous as it increases the mortality rate in patients with sepsis (Park et al. 2012) and in critically ill children (Faustino and Bogue 2010) and adults (NICE-SUGAR Study Investigators et al. 2012).

At present work we have shown that the combination of taurine and ethanol may be lethal to mice of different ages and its one possible reason is hypoglycemia. It seems that further studies on the mechanisms of ethanol and taurine induced hypoglycemia, mechanisms of hypoglycemic death after ethanol and taurine co-administration and possible other mechanisms associated need to be done in future.

5 Summary

In summary, this study showed that taurine alone administered even at high doses was safe for the animals. However, combination of high doses of taurine with alcohol has a toxic effect and can be lethal. Taurine alone lowered blood glucose in a dose-dependent manner but not to a dangerous level, suggesting a possible beneficial effect of taurine in the treatment of diabetes. The co-administration of taurine and ethanol also reduced blood glucose levels in a dose-dependent manner. The lethal doses of taurine

and ethanol combination in different age groups lowered the blood glucose level to approximately one half. However, 25 % of 7-day-old and 40 % of elderly mice treated with taurine and ethanol showed a dramatic drop in blood glucose to 2–3 mmol/l, which can be considered lethal. Hypoglycemia may thus be one cause of death after the co-administration of taurine and ethanol. However, since 100 % of animals died after the applied doses of taurine and ethanol, but only 25–40 % of them had dangerously low glucose levels, other mechanisms of toxicity must exist.

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Role of Taurine on the Actions of Alcohol Against Systemic and Cardiac Biochemical Changes in the Diabetic Rat

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Abbreviations

EtOH	Ethanol
TAU	Taurine
STZ	Streptozotocin
AST	Aspartate transaminase
CK	Creatine kinase
LDH	Lactate dehydrogenase
FA	Fatty acid
MDA	Malondialdehyde
GSH	Reduced glutathione
GSSG	Glutathione disulfide
CAT	Catalase
GPx	Glutathione peroxidase
SOD	Superoxide dismutase
IL-1β	Interleukin-1β
IL-6	Interleukin-6
TNF-α	Tumor necrosis factor-α
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate

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1 Introduction

Diabetic cardiomyopathy is a form of diabetes disease characterized by changes at the cellular and functional levels of cardiomyocytes and capable of progressing to structural, morphological and functional cardiac abnormalities that can lead to heart failure and arrhythmias (Battiprolu et al. 2010). The etiology of diabetic cardiomyopathy is not completely understood, but hyperglycemia and oxidative stress in cardiomyocytes are considered to play an important role in the pathogenesis. A unified mechanism that includes glucose autoxidation, increased production of advanced glycosylation end products, overproduction of superoxide anion radical ($O_2^{-\bullet}$) by the mitochondrial electron transport chain (ETC), and activation of the polyol and hexosamine pathways has been proposed to explain hyperglycemia-induced oxidative stress in cardiomyocytes (Brownlee 2005; Wang et al. 2013). Oxidative stress is thought to contribute to the initiation and progression of cardiac dysfunction and remodeling of the extracellular matrix in the heart.

Some of the mechanisms responsible for the cardiac dysfunction of this diabetic disease are increased expression of proinflammatory cytokines, accelerated myocardial injury, and metabolic changes such as increased lipolysis in adipocytes, increased free fatty acids (FFAs) in the circulation and cardiomyocytes, increased myocardial FA oxidation, and decreased myocardial glucose uptake and utilization (Abel 2005; Battiprolu et al. 2010). In addition there is impaired mitochondrial function, depletion of reduced glutathione (GSH), and altered mitochondrial morphology and integrity (Shen et al. 2004). With the help of chemical and transgenic animal models of type 1 and type 2 diabetes, it has been possible to demonstrate that myocardial insulin resistance is at the center of the mitochondrial dysfunction and the reduced ability of the heart to oxidize both glucose and FAs (Belke et al. 2002), with FAs serving as a substrate for building the intracardiac pool of triacylglycerols and ceramides, which together with unmetabolized FAs contribute to insulin resistance, cardiac lipotoxicity and the development of diabetic cardiomyopathy (Battiprolu et al. 2010; Ruberg 2007). In diabetes, an excessive accumulation of nonesterified FAs is detrimental to the cardiomyocyte in part by increasing oxygen demand, by contributing to mitochondrial uncoupling and the generation of reactive oxygen species (ROS) and the development of oxidative stress, one of the mechanisms responsible for mitochondrial dysfunction and decreased ATP synthesis (Abel 2005; Battiprolu et al. 2010).

Epidemiological studies suggest that a light to moderate ethanol (EtOH) consumption reduces the risk of developing cardiovascular diseases such as coronary artery disease, angina pectoris and myocardial infarction (Chen et al. 2003), improves survival after myocardial infarction, and can render the heart more tolerant to myocardial ischemia-reperfusion injury (Miyamae et al. 2010; Wang et al. 2007). Proposed mechanisms of cardioprotection by EtOH consumption have included the activation of adenosine A1 receptors, G-protein coupled α_1-adrenoceptors, protein kinase C isozymes, endothelial and inducible nitric oxide synthases (NOS), ATP-dependent potassium channels, decreased platelet aggregation, increases in high density lipoprotein cholesterol accompanied by decreases in

low density lipoprotein cholesterol, increased fibrinolytic activity, suppression of inflammatory cytokines, increases in insulin sensitivity and modifiable cardiac risks such as lipids and blood pressure, and activation of antioxidant enzymes such as catalase and superoxide dismutase (Miyamae et al. 2010).

Taurine (TAU) is an abundant endogenous product of the metabolism of methionine and cysteine endowed with cytoprotective activity in diabetes in general and in the diabetic heart in particular. There is experimental evidence to indicate that this sulfur-containing amino acid could be of benefit in diabetes by reducing the levels of blood glucose, glycated hemoglobin and plasma lipids, tissue lipid peroxidation and nitrosative injury, the loss of reduced glutathione, and the intracellular accumulation of Ca^{2+}, free radicals and other ROS produced during inflammatory responses (Roysommuti et al. 2003). In addition, TAU can decrease the formation of proinflammatory cytokines and of advanced glycation end products, the expression of chemokines, and the levels of neutrophil-generated hypochlorous acid (HClO) through formation of the less toxic and more stable taurine chloramine (TAU-Cl), a regulator of the severity of inflammatory responses and the activation of apoptotic pathways (Ito and Schaffer 2012; Roysommuti et al. 2003). There are also reports indicating that TAU can modulate mitochondrial Ca^{2+} handling, and prevent FA-decreased glucose-stimulated islet cell insulin secretion both in vivo and ex vivo, probably through an antioxidant action (Oprescu et al. 2007). On the other hand, in rats made diabetic with streptozotocin, the daily feeding of TAU, before and after the induction of the diabetes, was found to attenuate diabetes-induced alterations in cardiac function and cellular damage but without affecting the plasma levels of glucose, total cholesterol, triglycerides and high-density lipoprotein cholesterol as well as the myocardial accumulation of glycogen and lipids after a 11-week treatment period (Tappia et al. 2011). Moreover, the significant increase in cardiac TAU levels observed in diabetic cardiomyopathy may reflect a modulatory role for this amino acid on glycolysis (Militante et al. 2000).

Taking into account the known protective actions of EtOH and TAU on diabetes and/or the heart, this study was undertaken in a chemical rat model of diabetes to compare the abilities of these two agents in preventing diabetes-induced alterations of indices of glucose and lipid metabolism and of myocardial indicators of cell membrane damage, oxidative stress, inflammation and energy status. An additional purpose was to determine whether a combined treatment with EtOH and TAU can enhance the protective actions attained with the individual treatments.

2 Methods

2.1 Animals and Treatments

All the experiments were conducted with male Sprague-Dawley rats, 310–340 g in weight, randomly assigned to groups of six. Diabetes was induced with a single, 60 mg/kg, intraperitoneal dose of streptozotocin (STZ), dissolved in 10 mM citrate

buffer pH 4.5, on day 15. The diabetic rats were allowed to drink an EtOH solution (5 %, v/v, sweetened with Splenda® and flavored with a commercial sugar-free powdered lemonade product) in place of the drinking water from day 1 until day 28, with or without a concurrent treatment with TAU (2.4 mM/kg, by oral gavage). Control groups received only 10 mM citrate buffer pH 4.5, only STZ, only 5 % EtOH, only TAU or only 5 % EtOH plus TAU. All the treatments were terminated on day 28. The progress of the diabetes was monitored periodically by measuring the concentration of glucose in a drop of tail vein blood using a commercial glucometer. (TRUEtrack™ and test strips, Nipro Diagnostics, Fort Lauderdale, FL).

2.2 Samples

All the rats were sacrificed on day 29 of the study by cardiac puncture under isoflurane anesthesia. Blood samples were collected in heparinized tubes and centrifuged within 24 h at 700×*g* and 4 °C for 10 min to obtain their plasma fractions. Immediately thereafter, the animals were cut open and their hearts exposed and surgically excised using the freeze-clamp technique, and kept frozen in liquid nitrogen. A 0.5 g portion of frozen heart tissue was cut into small pieces with the help of a razor blade, placed in a polyethylene tube, mixed with ice-cold phosphate buffered saline (PBS) solution of pH 7.4, and homogenized using a hand-held electric tissue homogenizer (Tissue Tearor™, BioSpec Products, Inc., Bartlesville, OK). After bringing the volume to 10 mL with sufficient iced-cold PBS, the suspension was centrifuged at 3,000 rpm and 4° C for 30 min to obtain a clear supernatant which was withdrawn and kept on ice until needed.

2.3 Assays

2.3.1 Plasma Glucose

The plasma glucose was measured with a commercially available colorimetric assay kit (procedure No. 510, Sigma-Aldrich, St. Louis, MO) based on the method of Raabo and Terkildsen (1960). In the assay, the plasma sample was mixed with glucose oxidase, peroxidase and *ortho*-dianisidine, and the reaction is allowed to proceed at 37 °C for 30 min. The intensity of the resulting brown color was measured at 450 nm on a spectrophotometer. The results are expressed in mg/dL of plasma.

2.3.2 Plasma Insulin

An ELISA assay kit (Insulin ELISA, Calbiotech Inc., Spring Valley, CA) was used to measure the plasma insulin (INS) level. The results are expressed as μU/mL of plasma.

2.3.3 Plasma Cholesterol and Triglycerides

The total plasma cholesterol was measured by means of a commercial enzymatic-colorimetric cholesterol assay kit (Cholesterol LiquiColor®, Catalog No. 1010-430, Stanbio Laboratory, Boerne, TX) that is based on the method of Allain et al. (1974). The concentration of total (the sum of the esterified plus preformed free) cholesterol in the sample is expressed in mg/dL of plasma.

The levels of plasma and heart triglycerides were measured by means of commercial enzymatic-colorimetric assay kit (Enzymatic Triglycerides Procedure No. 2100 from Stanbio Laboratory, Boerne, TX) based on method of Fredrickson et al. (1967). The concentration of triglycerides in the sample is expressed in mg/dL of plasma.

2.3.4 Plasma Enzymatic Indices of Myocardial Damage

The plasma aspartate aminotransferase (AST) activity was measured according to the method of Bergmeyer et al. (1978) which is based on a set of two coupled reaction catalyzed by AST and malic dehydrogenase, respectively, and leading to the oxidation of NADH to NAD^+, which can be monitored spectrophotometrically at 340 nm. The results are expressed as U/L of plasma. The plasma lactate dehydrogenase (LDH) activity was measured by the method of Buhl and Jackson (1978), in which LDH catalyzes the oxidation of lactate to pyruvate with the concurrent reduction of NAD^+ to NADH. The rate of formation of NADH was followed spectrophotometrically by measuring the increase in absorbance at 340 nm. The results are expressed in U/L of plasma. The plasma creatine kinase (CK) activity was measured using a commercially available assay kit (CK Liqui-UV® Test catalog no. 2910-430, Stanbio Laboratory, Boerne, TX) based on a modification of the spectrophotometric method of Szasz et al. (1976) and in which *N*-acetyl-L-cysteine (NAC) is used to reactivate CK. The method determines the increase in absorbance at 340 nm due to the reduction of $NADP^+$ to NADPH. The results are expressed as U/L of plasma.

2.3.5 Plasma FFAs

The plasma concentration of FFAs was measured using a commercial microplate assay kit (Free Fatty Acid Quantification Colorimetric/Fluorometric Kit, BioVision Inc., Mountain View, CA). In the assay, the FFAs are converted to their coenzyme A derivatives which are subsequently oxidized with the concomitant generation of a color that can be quantified on a spectrophotometer at 570 nm. The results are expressed as nmol/mL of plasma.

2.3.6 Plasma and Heart Nonenzymatic Indices of Oxidative Stress

The extent of lipid peroxidation (LPO) in the plasma and heart was assessed by measuring the levels of malondialdehyde (MDA) as thiobarbituric acid reactive substances (TBARS) using the colorimetric end-point method of Buege and Aust

(1978). In this method, MDA is reacted with TBA to yield a pink chromophore whose absorbance can be measured spectrophotometrically at 535 nm. The results are expressed as nM/mL of plasma or as nM/g of tissue.

The content of reduced (GSH) and disulfide (GSSG) glutathione in the plasma and heart was measured with the fluorometric method of Hissin and Hilf (1976), which is based on the reaction of *ortho*-phthalaldehyde with GSH at pH 8 and with GSSG at pH 12.0. Prior to the measurement of GSSG, any interfering GSH is complexed with *N*-ethylmaleimide using the method of Güntherberg and Rost (1966). The concentrations of GSH and GSSG are expressed as μM/mL of plasma or as μM/g of tissue.

2.3.7 Plasma and Heart Enzymatic Indices of Oxidative Stress

The plasma and heart activities of catalase (CAT), glutathione peroxidase (GPx) and Cu, Zn superoxide dismutase (SOD) were measured according to the methods of Aebi (1984), Günzler and Flohé (1985) and Misra and Fridovich (1972), respectively. The results are expressed as U/min/mL of plasma or as U/min/g of tissue.

2.3.8 Heart Cytokines

The levels of heart interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) were measured using commercial microplate assay kits for rat tissue (Rat IL-1β ELISA Kit, Catalog No. ELR-IL/1β-001c, Rat IL-6 ELISA Kit, Catalog No. ELR-IL6-001c and Rat TNF-α ELISA Kit, Catalog No. ELR-TNF alpha-001/001c, obtained from RayBiotech Inc, Norcross, GA). A sample for each assay was prepared by pulverizing 50 mg of frozen heart tissue with the help of a mortar and pestle precooled in liquid nitrogen. The powder was immediately mixed with 1 mL of the sample diluent buffer included in the assay kit, and the suspension centrifuged at 3,000 rpm and 4 °C for 30 min. A 100 μL volume of heart homogenate was used in each assay as instructed by the manufacturer of the assay kit. The results are expressed as pg/g of tissue.

2.3.9 Heart ATP/ADP Ratio

The contents of adenosine triphosphate (ATP) and adenosine diphosphate (ADP) in the heart were measured using commercially available assay microplate assay kits (ATP Colorimetric/Fluorometric Assay Kit, Catalog No. K354-100 and ADP Colorimetric/Fluorometric Assay Kit, Catalog No. K355-100, both obtained from BioVision, Mountain View, CA). A lysate for the assay of ATP was homogenizing a 10 mg portion of heart tissue, kept on an ice bath, with 100 μL of ATP assay buffer with the help of a hand held tissue homogenizer, and subjecting the resulting suspension go centrifugation at 3,000 rpm and 4 °C for 10 min to obtain a clear

supernatant suitable for analysis. A sample for the assay of ADP was prepared in identical manner but using 100 µL of ADP assay buffer. A 50 µL volume of lysate was used in the assay. The samples were read on a microplate reader set at 570 nm after they were processed as suggested by the manufacturer of the kits. The concentrations of ATP and ADP are expressed in µM/g of tissue.

2.4 Statistical Analysis of Data

The experimental results are reported as mean ± standard error of the mean (SEM) for $n = 6$ rats. The results were analyzed for statistical significance using unpaired Student's *t*-test followed by one-way analysis of variance (ANOVA) and Tukey's *post hoc* test. Intergroup differences were considered to be statistically significant when $p \leq 0.05$.

3 Results

3.1 Plasma Glucose Levels

Figure 1 shows the changes in plasma glucose levels of treated and untreated diabetic rats. Diabetic rats exhibited a plasma glucose level that was more than fourfold higher ($p < 0.001$) than that of naive rats (127.03 ± 7.57 mg/dL). In contrast, the plasma glucose of diabetic rats drinking EtOH before the induction of diabetes was significantly lower (by 34 %, $p < 0.01$) than that of diabetic rats drinking plain water. A similar, although slightly greater, effect was observed in the presence of TAU (−42 %, $p < 0.001$ vs. diabetes). A further enhancement of this hypoglycemic action was possible by providing EtOH and TAU concurrently (−50 %, $p < 0.001$ vs. diabetes). Neither EtOH nor TAU altered the baseline plasma glucose to a significant extent.

3.2 Plasma INS Levels

As seen in Fig. 2, in diabetic rats the increase in plasma glucose was accompanied by a parallel significant decrease (−67 %, $p < 0.001$) of the plasma INS seen in normal rats (6.9 ± 1.2 µU/mL). The daily consumption of EtOH led to an effective attenuation of this effect (only −46 %, $p < 0.001$), which became $p < 0.01$. Allowing diabetic rats to consume EtOH in the presence of TAU led to a further reduction of the decrease in plasma INS brought about by diabetes (−26 %, $p < 0.01$) than was possible with the individual treatments. Neither EtOH nor TAU altered the baseline plasma INS to a significant extent.

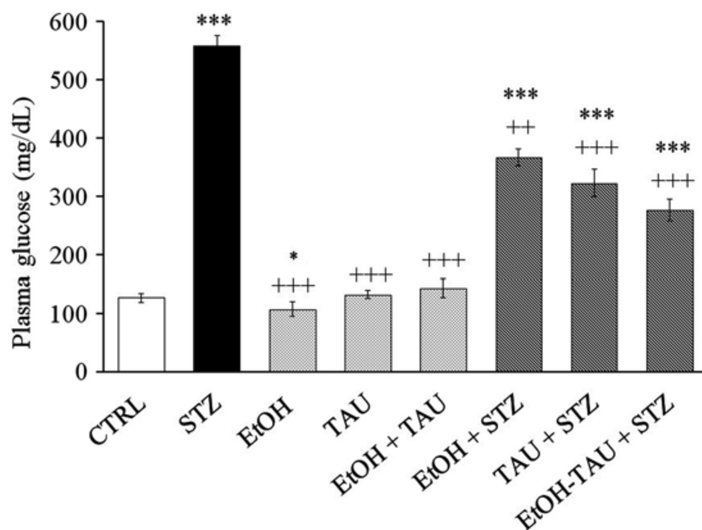


Fig. 1 The effects of EtOH, TAU and TAU-EtOH on the plasma glucose levels of diabetic rats. Results are shown as mean ± SEM for $n = 6$. Differences were significant vs. control (CTRL) at $^{*}p < 0.05$ and $^{***}p < 0.001$; and vs. diabetes (STZ) at $^{++}p < 0.01$ and $^{+++}p < 0.001$

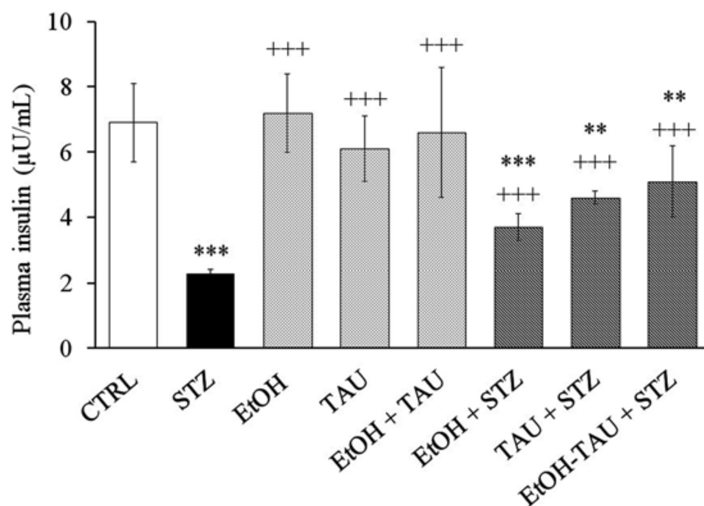


Fig. 2 The effects of EtOH, TAU and TAU-EtOH on the plasma INS levels of diabetic rats. Results are shown as mean ± SEM for $n = 6$. Differences were significant vs. control (CTRL) at $^{**}p < 0.01$ and $^{***}p < 0.001$; and vs. diabetes (STZ) at $^{+++}p < 0.001$

3.3 Plasma Cholesterol and Triglycerides Levels

The results for the plasma concentrations of cholesterol and triglycerides for the various experimental groups are displayed in Fig. 3a, b, respectively. Diabetes markedly elevated the circulating cholesterol level (+61 %, $p<0.001$) over the

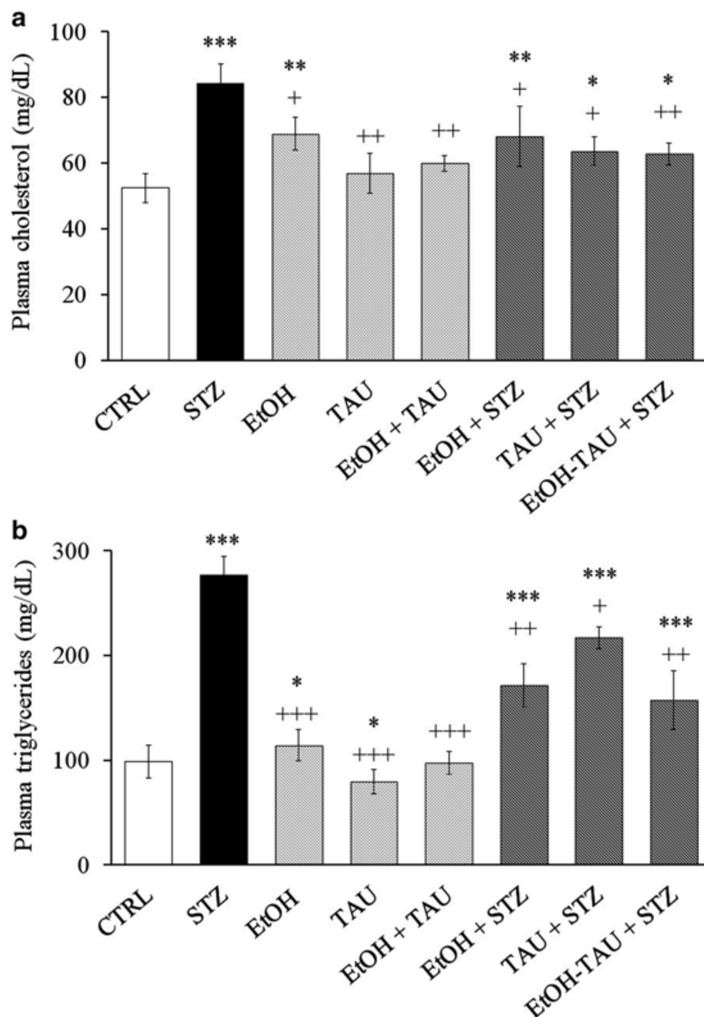


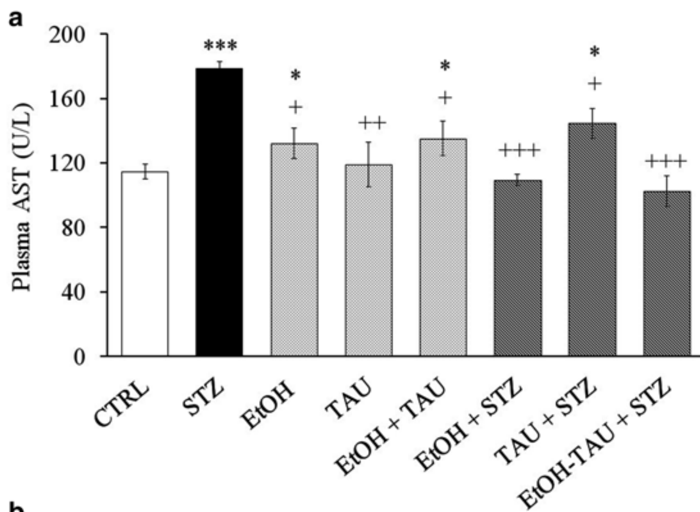
Fig. 3 The effects of EtOH, TAU and TAU-EtOH on the plasma levels of (**a**) cholesterol and (**b**) triglycerides of diabetic rats. Results are shown as mean ± SEM for $n=6$. Differences were significant vs. control (CTRL) at $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$; and vs. diabetes (STZ) at $^{+}p<0.05$, $^{++}p<0.01$ and $^{+++}p<0.001$

control value (84.22 ± 5.89 mg/dL) (Fig. 3a). This effect was significantly attenuated in rats consuming EtOH (+30 %, $p < 0.01$) or receiving TAU (+22 %, $p < 0.05$) daily. On the other hand a combined treatment with EtOH plus TAU with equipotent to a treatment with TAU alone. The plasma triglyceride levels (Fig. 3b) followed a similar trend to those seen with the cholesterol levels. In diabetic rats the plasma triglycerides was greatly (+180 %, $p < 0.001$) relative to control rats (98.87 ± 15.74 mg/dL). Allowing the rats to start consuming EtOH before the induction of diabetes lead to a significant attenuation of this effect (−38 %, $p < 0.001$ vs. diabetes). A similar preconditioning with TAU also reduced the increase but to a lower extent (−22 %, $p < 0.05$ vs. diabetes); and one with EtOH plus TAU was not significantly different from one with EtOH alone (−43 %, $p < 0.001$ vs. diabetes) (Fig. 3b).

3.4 Plasma AST, CK and LDH Activities

The occurrence of cardiac injury was investigated by measuring the activities of AST, CK and LDH in the plasma. As shown in Fig. 4a, the diabetic value for plasma AST rose significantly (by ~55 %, $p < 0.001$) over the control value (114.54 ± 4.59 U/L). This change was virtually abolished by the intake of EtOH (only +4 %), and significantly reduced by a treatment with TAU (+26 %, $p < 0.01$). A combined treatment with EtOH plus TAU reduced the elevation to a value not significant different from the control value (+11 %). Similarly, the data presented in Fig. 4b indicates that diabetes caused a massive increase in plasma CK (+75 %, $p < 0.001$) compared to the value for control rats (1,424.30 ± 35.76 U/L). The intake of EtOH or a treatment with TAU resulted in a strong reducing effect on this increase (only +15 % and +21 %, respectively, both at $p < 0.05$ vs. control), an effect that was accentuated when these two agents were provided alongside. Diabetes also promoted the release of LDH into the circulation (+53 %, $p < 0.001$) although to a lesser extent than those seen with AST and CK (Fig. 4c). EtOH and TAU, alone or in combination, reduced the release (<10 %) to values not significant different from the control value (426.22 ± 26.64 U/L). Except for a small increase in the plasma AST activity by EtOH (+18 %, $p < 0.05$), neither EtOH nor TAU, alone or together, had a significant elevating effect on the baseline values of CK and LDH.

Fig. 4 The effects of EtOH, TAU and TAU-EtOH on the plasma activities of (**a**) AST, (**b**) CK and (**c**) LDH of diabetic rats. Results are shown as mean ± SEM for $n = 6$. Differences were significant vs. control (CTRL) at $^{*}p < 0.05$ and $^{***}p < 0.001$; and vs. diabetes (STZ) at $^{+}p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$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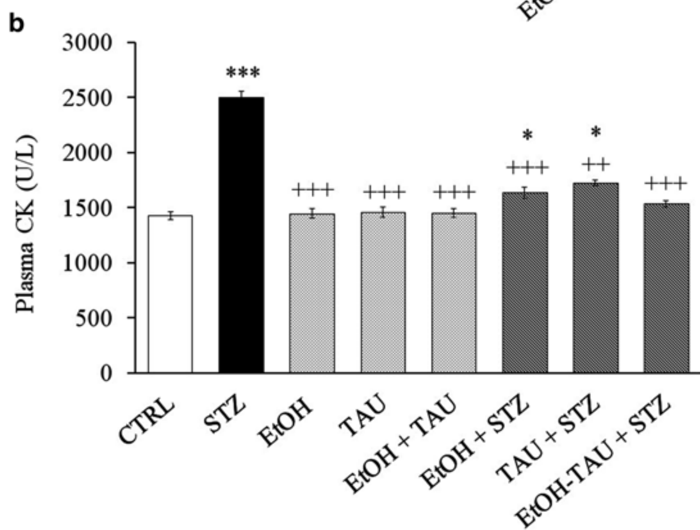
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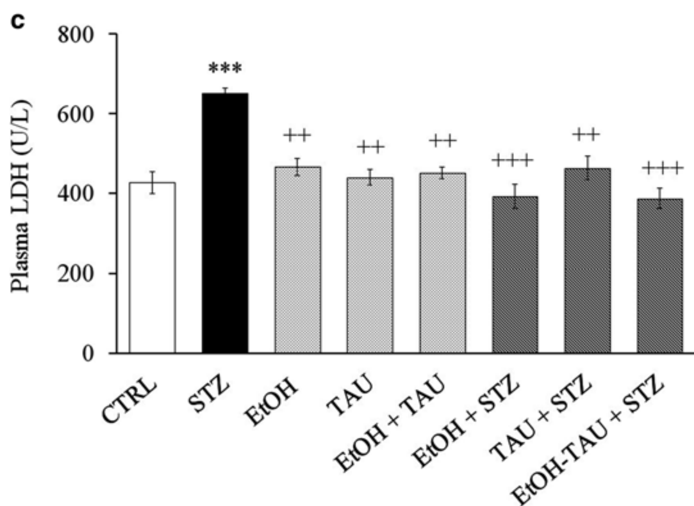
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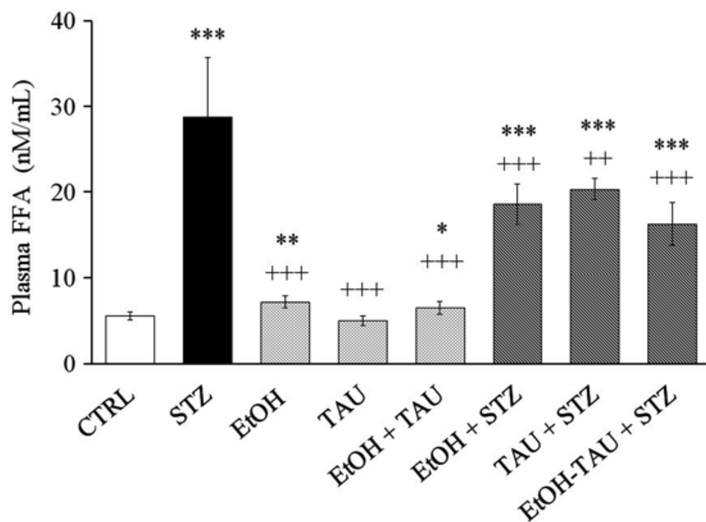

Fig. 5 The effects of EtOH, TAU and TAU-EtOH on the plasma FFAs levels of diabetic rats. Results are shown as mean ± SEM for $n = 6$. Differences were significant vs. control (CTRL) at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$; and vs. diabetes (STZ) at $^{+}p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$

3.5 Plasma FFAs Levels

As shown in Fig. 5, the plasma of diabetic rats contained much higher levels of plasma FFAs levels (>410 %, $p < 0.001$) than the plasma from control rats (5.46 ± 0.44 nM/mL). This increase was reduced significantly in diabetic rats consuming EtOH (+235 %) or receiving TAU (+265 %) on a daily basis (both at $p < 0.001$ vs. diabetes). The same figure also indicates that a conditioning with EtOH plus TAU was more effective than either agent alone (only +190 %, $p < 0.001$ vs. diabetes). In naive rats, EtOH, alone or in the presence of TAU, showed a significant elevating effect on the plasma FFAs (≥18 %, $p \leq 0.05$ vs. control).

3.6 Plasma and Heart MDA Levels

As shown in Fig. 6a, the concentration of MDA was significantly higher in the plasma and heart of diabetic rats (both by 29 %, $p < 0.01$) than in control rats (11.24 ± 0.18 nM/mL and 136.50 ± 2.36 nM/g, respectively). In rats receiving either EtOH or TAU, the formation of MDA was significantly lower than in diabetic rats both in the plasma (by 19 % and 22 %, respectively, both at $p < 0.05$ diabetes) and in the heart (by 16 %, $p < 0.05$, and 32 %, $p < 0.01$, respectively). A combined treatment of with EOH and TAU, on the other hand, reduced the diabetic concentrations

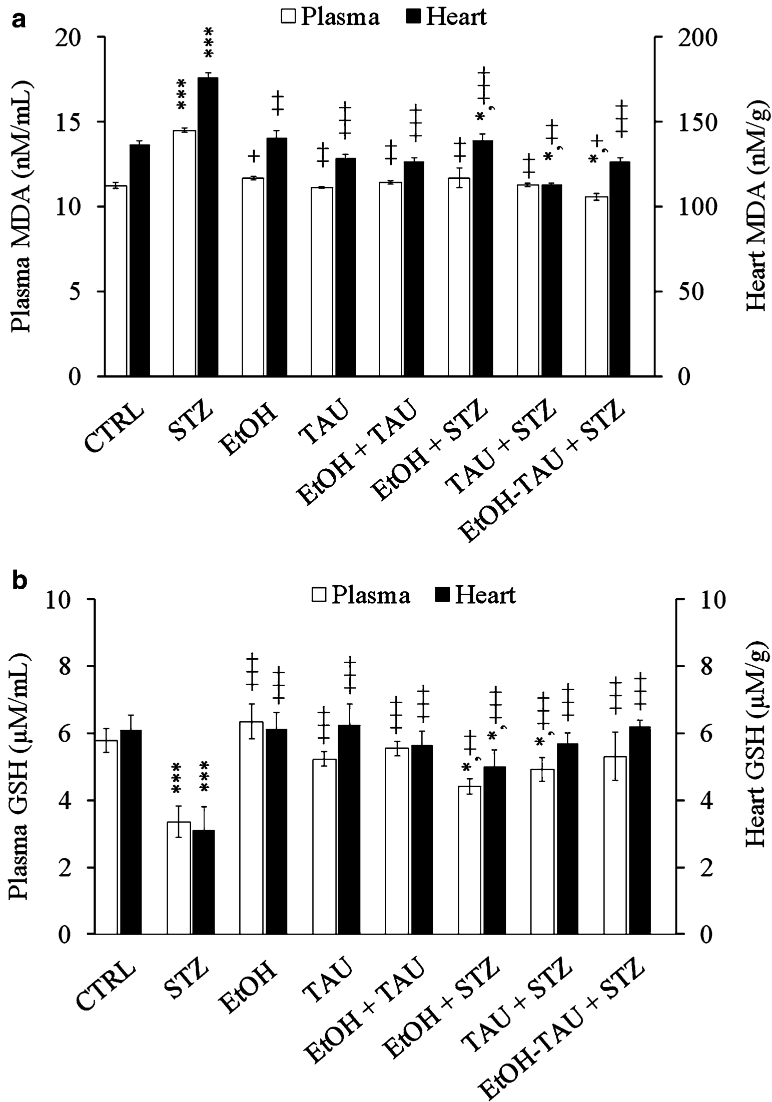

Fig. 6 The effects of EtOH, TAU and TAU-EtOH on the plasma and heart levels of MDA, GSH and GSSG. Results are shown as mean ± SEM for $n = 6$. Differences were significant vs. control (CTRL) at $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$; and vs. diabetes (STZ) at $^{+}p<0.05$, $^{++}p<0.01$ and $^{+++}p<0.001$

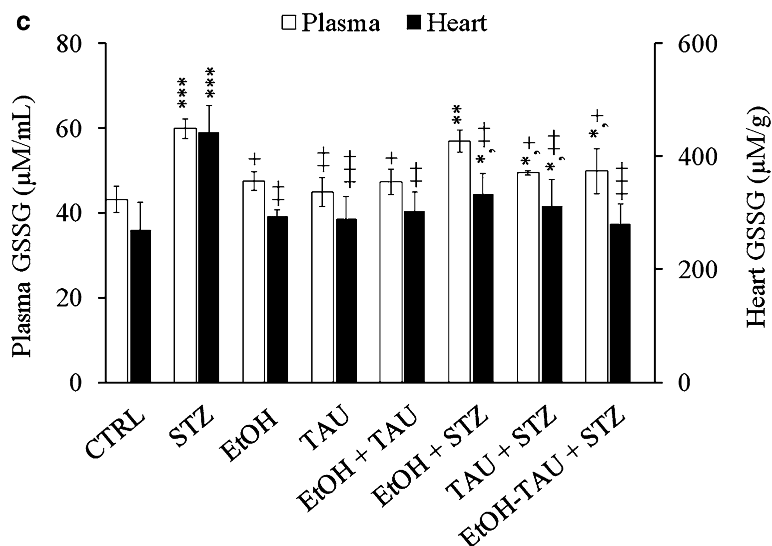

Fig. 6 (continued)

of MDA in the plasma (by 27 %, $p<0.01$) and heart (by 24 %, $p<0.05$) to values that were closer to those seen with TAU than with EtOH.

3.7 Plasma and Heart GSH and GSSG Levels

The results for the assays of the plasma and heart GSH are shown in Fig. 6b. Diabetic rats showed lower plasma and heart GSH levels (by 42 % and 49 %, respectively, $p<0.001$) than corresponding control values (5.78±0.36 µM/mL and 6.09±0.44 µM/g, respectively). The intake of EtOH by diabetic rats lowered the losses of GSH in the plasma and heart significantly (only −24 % and −18 %, respectively, both at $p<0.05$ vs. controls). In both instances, a daily treatment with TAU appeared more potent than one with EtOH (−15 % and +4 %, respectively). On the other hand, making EtOH and TAU concurrently available to the diabetic rats reduced the losses in plasma and heart GSH to values not different from control values. In naïve rats, neither EtOH nor TAU was found to exert an appreciable effect on the baseline GSH values.

From the results presented in Fig. 6c, it is apparent that diabetes caused an elevation of the plasma (by ~40 %, $p<0.001$) and heart (by 64 %, $p<0.001$) GSSG contents seen in control normal rats (43.16±3.14 µM/mL and 269.61±48.74 µM/g, respectively). Allowing the diabetic rats to drink EtOH reduced both increases to different extents (+32 %, $p<0.01$, and +23 %, $p<0.05$, respectively). The administration of TAU in place of EtOH resulted in a small gain in attenuating action (+15 % and +16 %, respectively); and a co-treatment with EtOH plus TAU led to a plasma

GSSG similar to that seen in the presence of TAU alone and a heart GSSG level similar to control. None of the treatment agents demonstrated a significant positive effect on the plasma and heart GSH and GSSG levels (Fig. 6c).

3.8 Plasma and Heart CAT, GPx and SOD Activities

A further insight into the development of oxidative stress was obtained by measuring the activities of the antioxidant enzymes CAT, GPx and SOD in the plasma and heart of diabetic rats. The data presented in Fig. 7a–c indicate that diabetes reduced the activities of the three enzymes in both samples to a significant extent. In diabetic rats, the plasma and heart CAT activities were much lower (−50 %, $p<0.001$, and 41 %, $p<0.001$, respectively) than corresponding control values (91.28 ± 2.18 U/min/mL and 7.46 ± 0.38 U/min/mg, respectively) (Fig. 7a). These effects were attenuated appreciably in the presence of a daily intake of EtOH (−33 %, and −31 %, respectively, both at $p<0.01$ vs. control). A similar treatment schedule with TAU afforded a greater protection (−10 % and −15 %, respectively); but one with EtOH plus TAU was only slightly better than one with TAU alone (8 % and 2 % decreases).

Diabetes also lowered the activity of GPx both in the plasma (by 36 %, $p<0.01$) and heart (by 54 %, $p<0.001$) (Fig. 7b). As observed with CAT, the intake of EtOH by diabetic rats lessened the decreases in activity (by only 19 %, $p<0.05$, and 13 %, respectively). While a similar degree of protection was obtained derived from a treatment with TAU (−12 % and −17 %, $p<0.05$), one with EtOH plus TAU was no different in potency than one with TAU alone.

The data reported in Fig. 7c is also consistent with a lowering by diabetes of the plasma and heart activities of SOD (by 38 % in both, $p<0.001$) of control animals. The daily consumption of EtOH by the diabetic rats resulted in a significant attenuation of these decreases (–7 % and –20 %, $p<0.05$, respectively, vs. controls). Providing TAU in place of EtOH also resulted in effective attenuation of the SOD activity loss, particularly in the heart (–6 % and –8 %, respectively), but which was not significant different from the effects of EtOH. On the other hand, a co-treatment with EtOH and TAU was no different in potency from one with TAU alone (–4 % and –8 %, respectively).

Except for a small decrease in heart CAT activity (−18 %, $p<0.05$) cause by EtOH, none of the treatment agents or their combination demonstrated a significant effect on the baseline activities of the antioxidant enzymes in the plasma and heart (Fig. 7a–c).

3.9 Heart ATP and ADP Levels and ATP/ADP Ratios

The existence of an abnormal myocardial energy metabolism as a result of diabetes was investigated by measuring the cardiac levels of ATP and ADP. From the results presented in Fig. 8, it is evident that the heart of diabetic rats contained less ATP

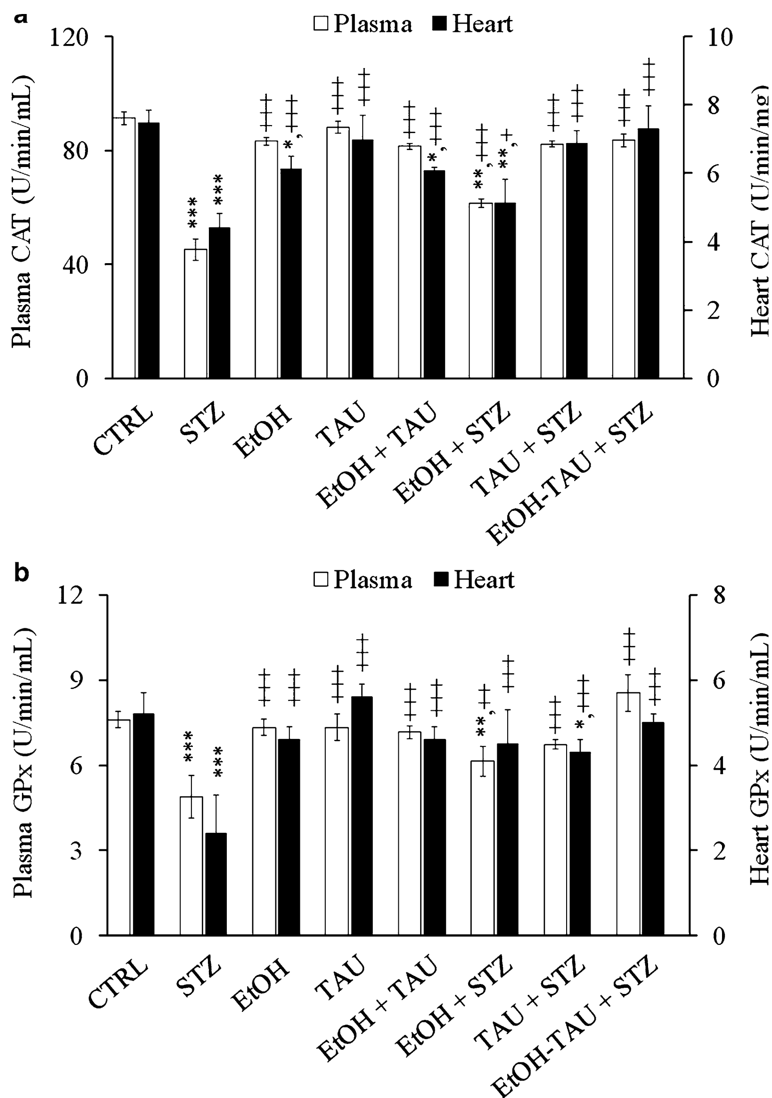

Fig. 7 The effects of EtOH, TAU and TAU-EtOH on the plasma and heart activities of (**a**) CAT, (**b**) GPx and (**c**) SOD of diabetic rats. Results are shown as mean ± SEM for $n = 6$. Differences were significant vs. control (CTRL) at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.01$; and vs. diabetes (STZ) at $^{+}p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$

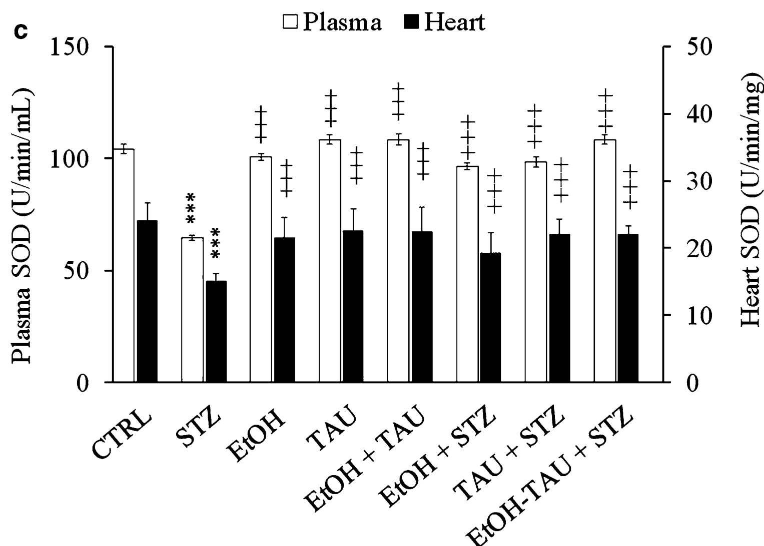

Fig. 7 (continued)

(−24 %, $p<0.05$), more ADP (+40 %, $p<0.001$) than hearts of naive rats (40.91 ± 1.55 μM/g and 20.55 ± 1.01 μM/g, respectively) and, hence, a lower ATP/ADP ratio (1.08 vs. 1.99). Allowing the diabetic rats to drink EtOH in place of plain water led to a marked reduction in the loss of ATP (only −14 %), gain of ADP (+6 %), and ensuing decrease in the ATP/ADP ratio (−19 %, $p<0.05$). In this regard, treating the diabetic rats with TAU rather than with EtOH led to a reversal of the effect of diabetes on ATP (+6 %), a lower ADP accumulation (+17 %, $p<0.05$), and a higher ATP/ADP ratio than untreated diabetic rats (1.80). On the other hand, the effect of a combined treatment of diabetic rats with EtOH plus TAU on the myocardial ATP (−3 %), ADP (+17 %, $p<0.05$) and ATP/ADP ratio (1.7) was rather similar to one with TAU. Neither EtOH nor TAU, alone or in combination, altered the myocardial ATP/ADP ratio significantly (Fig. 8).

3.10 Heart Proinflammatory Cytokines

The occurrence of inflammation in the diabetic heart was investigated on the basis of the cardiac levels of proinflammatory cytokines known to be associated with coronary heart disease, namely IL-1β, IL-6 and TNF-α (Yudkin et al. 2000). From the results shown in Fig. 9a–c it is evident that the heart of diabetic rats contained higher levels of IL-1β (+100 %), IL-6 (+77 %) and TNF-α (+135 %) than normal hearts (1,593.85 ± 42.09 pg/g, 1,622.12 ± 175.21 pg/g and 1,314.67 ± 143.33 pg/g,

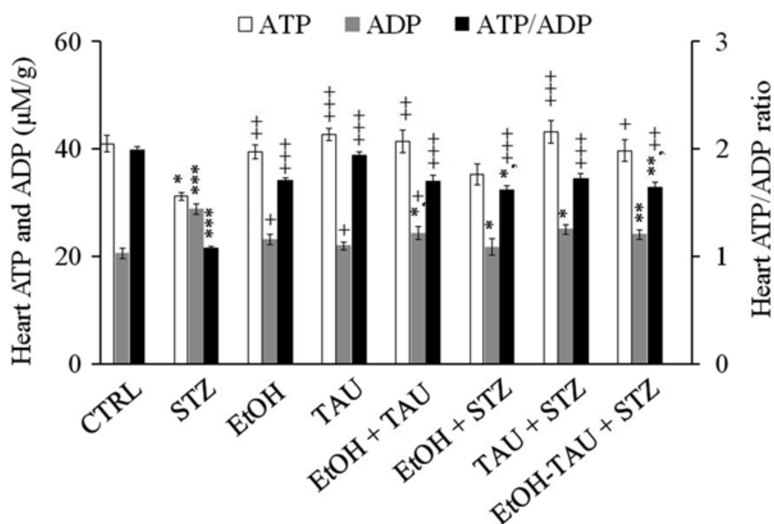

Fig. 8 The effects of EtOH, TAU and TAU-EtOH on the plasma and heart (**a**) ATP and (**b**) ADP levels and on (**c**) ATP/ADP ratio of diabetic rats. Results are shown as mean ± SEM for $n = 6$. Differences were significant vs. control (CTRL) at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$; and vs. diabetes (STZ) at $^{+}p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$

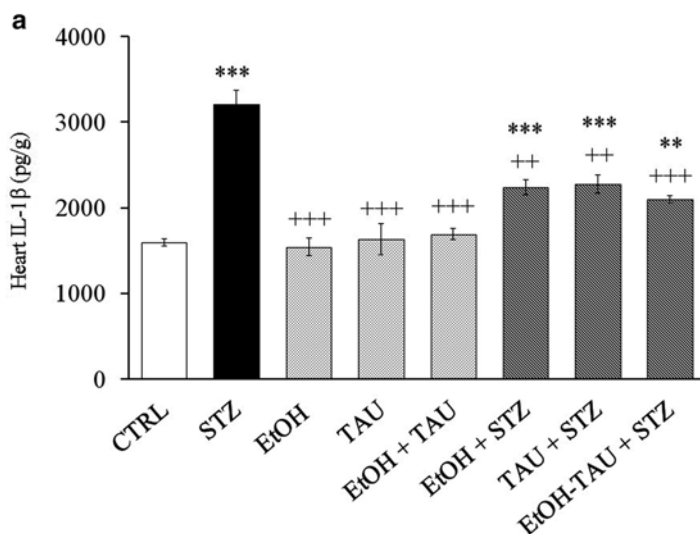

Fig. 9 The effects of EtOH, TAU and TAU-EtOH on the heart levels of (**a**) IL-1β, (**b**) IL-6 and (**c**) TNF-α of diabetic rats. Results are shown as mean ± SEM for $n = 6$. Differences were significant vs. control (CTRL) at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$; and vs. diabetes (STZ) at $^{+}p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$

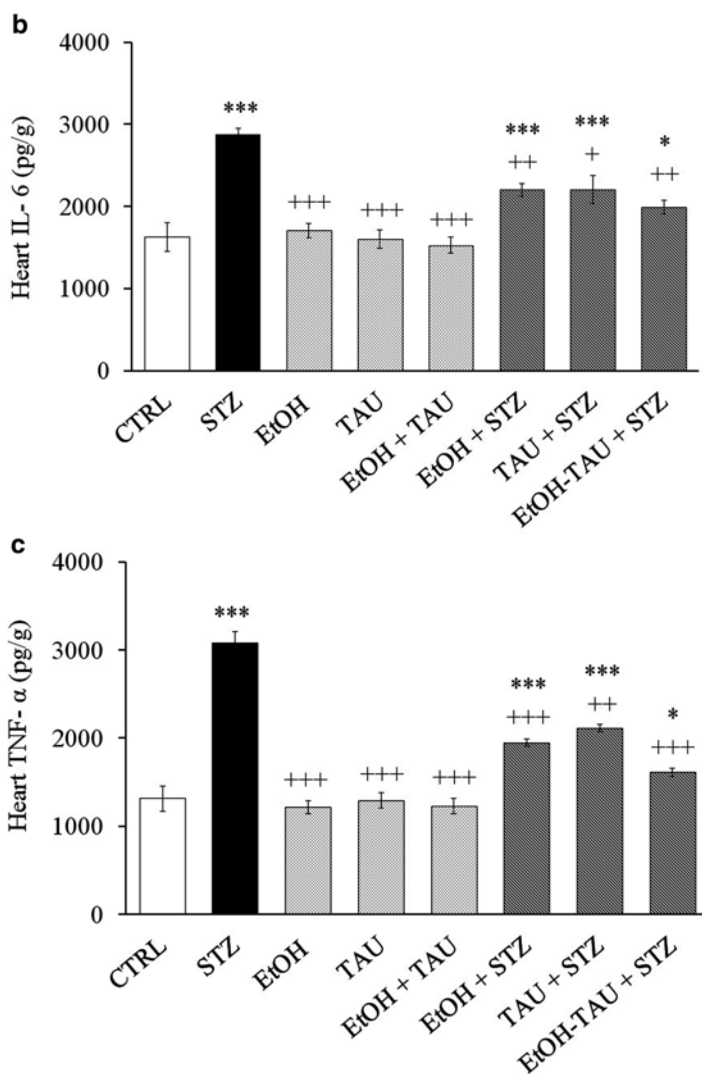

Fig. 9 (continued)

respectively, all at $p<0.001$). The same figures also indicate that the daily consumption of a low concentration of EtOH was effective in reducing these elevations (increases of only 41 %, 36 % and 48 %, respectively, $p<0.001$ vs. controls). In comparison with EtOH, a similar treatment schedule with TAU was somewhat less effective against the increase in IL-6 (+36 %, $p<0.001$) and TNF-α (+60 %, $p<0.001$) and about equipotent against the increase in IL-1β (+43 %, $p<0.001$). However, providing TAU and EtOH concurrently led to greater attenuating effect

against increases of all three cytokines (increases of 32 %, 23 % and 23 %, respectively, $p \leq 0.05$ vs. controls).

4 Discussion

Preconditioning is an endogenous adaptive protective response to a deleterious insult by cells, tissues or organs following a brief exposure to a physicochemical stress (Kuhlmann et al. 1997), to a low to moderate concentration of an exogenous substance (Collins et al. 2009) or as a result of an appropriate environmental adjustment (Wang et al. 2013). Among preconditioning agents, EtOH is probably the most widely used in situations where cell injury such as ischemic-reperfusion cardiac injury (Chen et al. 2003; Miyamae et al. 1997; Collins et al. 2009), renal injury (Fan et al. 2012) or inflammation (Sierksma et al. 2002) are of concern. In the present study, preconditioning was carried out by continuously making EtOH available in the drinking water and starting at 2 weeks prior to the induction of diabetes with STZ. The concentration of the EtOH solution used (5 % v/v) is one reported to improve survival and to reduce cardiomyocyte damage in guinea pigs later developing a myocardial infarction (Miyamae et al. 1997). Using the same treatment schedule, TAU was provided by oral gavage at a dose (2.4 mM/kg) known in this laboratory to be effective in curtailing metabolic and biochemical changes associated with STZ-induced diabetes in the rat (Pandya et al. 2010).

Irrespective of the metabolic parameter examined, both EtOH and TAU were able to attenuate the hyperglycemia, hypoinsulinemia, dyslipidemia and increased lipolysis seen in diabetic rats. The effects were found to be not only invariably statistically significant but to follow a common pattern. However, when comparing the individual potencies, the attenuating effect was in all instances, but on lipolysis, greater with TAU than with EtOH. The possibility that EtOH and TAU could be exerting their protective effects through different mechanism was suggested by the results of experiments in which these agents were used concurrently and which found the combination to be more effective than the individual treatments. Moderate ingestion of EtOH in type 2 diabetics are associated with reduced fasting INS level and improved INS sensitivity, which also translates into reduced dyslipidemia (Nogueira et al. 2014). In high concentrations EtOH is known to cause hypoglycemia by inhibiting hepatic gluconeogenesis, which may be of concern in the absence of adequate dietary intake (Badawy 1977). The lowering of the plasma FFAs by 5 % EtOH confirms the report that consumption of low dose EtOH by young healthy male volunteers leads to a modest activating action on hepatic de novo lipogenesis and hypertiglyceridemia, and that its hepatic metabolism generates acetate for transport to peripheral tissues where it inhibits lipolysis (Feinman and Lieber 1999). The beneficial effects of TAU on parameters of glucose metabolism are consistent with its reported protective effects against diabetes-induced β-cell damage (Chang and Kwon 2000) and apoptosis (Lin et al. 2013), against FA-mediated decrease in

glucose-stimulated INS secretion and islet ROS production (Oprescu et al. 2007), and ability to enhance peripheral glucose uptake in INS-resistance states (Haber et al. 2003).

The significance increases in plasma AST, total CK and LDH activity values is taken as an indication that diabetes can cause myocardial injury. In this case, the trend of the increases (i.e. total CK>AST>LDH) is akin with that reported for human patients experiencing chest pains during a myocardial infarct (Moss et al. 1986). Preconditioning with either EtOH or TAU reduced these elevations to values that were not very difference from those of naïve animals. As a result, protection by EtOH and TAU was about equal and protection by their combination was only insignificantly better than the individual treatments. Since in type 2 diabetes, myocardial injury is regarded as a consequence of oxidative stress arising directly or indirectly from hyperglycemia, hyperlipidemia, hyperinsulinemia, and INS resistance, alone or in combination (Ansley and Wang 2013), the protective role of EtOH and TAU may be tied up, at least in part, to their inherent antioxidant activities (Ito and Schaffer 2012) and, in terms of TAU, also to a membrane stabilizing effect (Roysommuti et al. 2003). At low concentrations, the metabolism of EtOH generates nicotinamide adenine dinucleotide for creating a reductive environment that decreases oxidative stress and the secondary production of aldehydes through LPO, and for maintaining the intracellular cysteine and GSH in their reduced state (Vasdev et al. 2006). Indeed, in the present work and that of other laboratories (Ansley and Wang 2013; Kumawat et al. 2013; Maritim et al. 2003), the development of diabetes is found to be accompanied by increases in MDA, a marker of lipid peroxidation (LPO), in the plasma and heart tissue, by a drop in the corresponding GSH/GSSG ratios, and by lower circulating and cardiac activities of antioxidant enzymes participating in the removal of hydrogen peroxide, organic peroxides and superoxide anion radicals ($O_2^{-\bullet}$). A direct lowering of free radicals and other ROS by TAU has been a subject of a long debate since its molecule lacks a readily oxidizable functionality and antiradical activity in cell-free radical-generating systems has been weak to nil (Aruoma et al. 1998; Tadolini et al. 1995). An indirect antioxidant role seems to underline some of the actions of TAU, which includes the enhancement of expression and activities of antioxidant enzymes (Jang et al. 2009) and the binding of reactive aldehydes including glucose (Ogasawara et al. 1993). In the diabetic heart in particular, the occurrence of oxidative stress has been correlated with increases in $O_2^{-\bullet}$ levels, LPO and activity of NADPH oxidase (Ansley and Wang 2013), with decreases in antioxidant enzyme defenses, and with the leakage of electron and $O_2^{-\bullet}$ from a dysfunctional myocardial mitochondrion (Ansley and Wang 2013). Hyperglycemia is the driving factor in $O_2^{-\bullet}$ overproduction in the mitochondrion by causing the disruption of the ETC, activating NADPH oxidase and uncoupling NOS (Giacco and Brownlee 2010). While a depletion of TAU by hyperglycemia may contribute to mitochondrial dysfunction (Hansen et al. 2010) and apoptotic myocardial cell death (Jong et al. 2011), a supplementation with it is found to reduced mitochondrial LPO and $O_2^{-\bullet}$ generation in the oxidatively stressed heart under diabetic (Schaffer et al. 2009) and nondiabetic (Parvez et al. 2008) conditions.

The role of inflammation in the pathogenesis and progression of myocardial injury in chronic diabetes is well established. The injurious effect of chronic diabetes in the myocardium could result from either a persistent inflammatory signaling directly to the heart or from the dysregulation of antiinflammatory signaling systems (Drimal et al. 2008). Irrespective of its etiology, an inflammatory component of diabetes leading to cardiac damage and dysfunction is the release of proinflammatory cytokines such as IL-1β, IL-6 and TNF-α and of chemoattractants from inflammatory cells in chemically-induced diabetes in mice (Chao et al. 2009) and rats (Drimal et al. 2008; Jain et al. 2007). In the present work, the cardiac levels of these three cytokines were dramatically increased relative to levels recorded for nondiabetic rats, with those of TNF-β predominating over those of IL-1β and Il-6 in that order. Both EtOH and TAU drastically suppressed these elevations, especially when provided alongside, with the degree of the effect varying inversely to the degree of proinflammatory cytokine elevation. The present effects of EtOH, representing a light and short consumption, contrast markedly with those observed during prolonged heavy consumption and in which it can trigger the recruitment and activation of inflammatory cells (Szabo et al. 2007). In contrast, results of in vitro studies in which monocytes were exposed to moderate concentration of EtOH found IL-6 release to be decreased via a transient decrease in the activity of the transcription factor nuclear factor-kappa B (NF-κB) (Mandrekar et al. 1997); and human intervention studies with moderate EtOH intake have verified a decrease inflammatory response via an increase in circulating adinopectin, an adipocyte protein that enhances insulin sensitivity by increasing FA oxidation and inhibiting hepatic glucose production (Lihn et al. 2005). Moreover, EtOH is able to induce the antiinflammatory cytokine IL-10 that can cause long lasting disruption of proinflammatory, cytokine-yielding, cascades (Qin et al. 2008). The characteristic natural abundance of TAU in cells with a high capacity for generating oxidants (i.e., neutrophils, macrophages) heralds an important protective role in oxidative stress-related inflammation. However, its antioxidant action is exerted only after conversion by neutrophil myeloperoxidase to TAU-Cl upon condensation with HOCl (Marcinkiewicz and Kontny 2014). TAU-Cl is a strong modulator of the immune system, capable of down regulating the production of proinflammatory cytokines in human and rodent leukocytes by inhibiting the activation of NF-κB, a potent signal transducer for proinflammatory cytokine expression (Schuller-Levis and Park 2004).

To be able to sustain its physiological functions, basal metabolic functions and ion-modulating actions, the heart needs a continuous supply of energy in the form of ATP, most of which is derived from mitochondrial β-oxidation of FA and the oxidative phosphorylating activity of the ETC, with the rest coming from glycolysis and guanosine triphosphate formed in the citric acid cycle (Lopaschuk et al. 2010). In the present study, the energy status of the diabetic heart was assessed by measuring the changes in ATP and ADP concentrations and calculating the corresponding ratios. Evidence of mitochondrial dysfunction was suggested by the extensive decrease in the value of the ATP/ADP ratio. Both EtOH and TAU were found to be effective in limiting this decrease, making the value barely significant when compared to the baseline value. Moreover, the effect of a co-treatment with these agents

was not significantly different from that attained with each agent alone. In the case of EtOH, the concentration of the EtOH solution seems to be a critical determining factor of the trend of changes in adenosine nucleotides in the heart. In this study, free intake of a low (5 %) concentration of EtOH had only a small decreasing effect on the heart ATP/ADP ratio, solely as a result of a small increase in ADP. On the other hand, the oral intake of 7.5 % EtOH (equal to 1 g/kg) was without effect on ATP, ADP and inorganic phosphate levels in the rat liver (Lindros and Stowell 1982). In contrast; treating rats with an intraperitoneal 1.5 g/k dose of EtOH elicited a slight increase in the ATP level 1 h later but without altering the levels of other adenine nucleotides (Pösö and Forsander 1976). In high doses and a long exposure, however, EtOH can reduce mitochondrial GSH, a cofactor for enzymes reducing hydrogen peroxide and organic peroxides, thus making this organelle more susceptible to oxidative damage and the development of LPO, and unable to carry out oxidative phosphorylation and ATP synthesis (Fernández-Checa and Kaplowitz 2005). In this context, TAU has proven of benefit in preventing excessive generation of ROS by the ETC (Schaffer et al. 2014), and in suppressing LPO and enhancing antioxidant enzyme activity in the myocardium (Wang et al. 2013). At present, the exact mechanism whereby diabetes causes mitochondrial dysfunction in the heart is incompletely understood since more than one factor seems to be involved. In addition to oxidative stress, hyperglycemia, increased uptake of FFAs, hyperinsulinemia and insulin resistance may all play a role (Abel 2005; Ansley and Wang 2013). Hyperglycemia is found to disrupt the ETC, to activate NADPH oxidase to produce $O_2^{-\bullet}$, and to uncouple NOS, a problem that compounds the oxidative stress since more $O_2^{-\bullet}$ and less NO is produced (Ansley and Wang 2013). Increased FFAs not only stimulates NADPH oxidase and decreases intracellular GSH but also contributes to the formation of di- and triacylglycerols and ceramides, the accumulation of which has been implicated in the development of INS resistance, cardiac dysfunction and cardiac failure (Lopaschuk et al. 2010). In turn, INS resistance can increase myocardial oxygen utilization and FA utilization and oxidation to ROS for eventual oxidative stress and cell damage. $O_2^{-\bullet}$ is of particular importance in cardiac energetics since it can uncouple ATP synthesis from oxidative metabolism directly by activating uncoupling proteins and indirectly via formation of LPO products (Lopaschuk et al. 2010). In addition, the use of proteomics has established a correlation between a deficiency of INS in diabetes with increased nitration of mitochondrial proteins and with downregulation of genes encoding for specific protein components of the ETC (Abel 2005).

5 Conclusions

Preconditioning with either a low-concentration EtOH solution or TAU was found to effectively protect the diabetic rat heart in a similar manner against diabetes-related biochemical alterations relevant to the pathogenesis of diabetic cardiomyopathy. Although differences in potency between the two treatment agents were

apparent, they were generally nonsignificant. However, providing EtOH and TAU together generally resulted in a greater protection than that attained with the individual treatments.

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Long-Term Taurine Supplementation Leads to Enhanced Hepatic Steatosis, Renal Dysfunction and Hyperglycemia in Mice Fed on a High-Fat Diet

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Abbreviations

T2D	Type 2 Diabetes
TAU	Taurine
HFD	High fat diet
KiTT	Glucose decay constant
Akt	Protein kinase B
FAS	Fatty acid synthase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

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1 Introduction

The incidence of Type 2 Diabetes (T2D) is a major public health issue that parallels the growing prevalence of overweight and obesity (Haffner 2006). Insulin resistance is a metabolically deranged state that precedes T2D and is hallmarked by increased hepatic glucose output and impaired glucose uptake by muscle cells and adipocytes (Schenk et al. 2008). Importantly, poor glucose control is associated with ectopic lipid accumulation in the liver, kidneys and other tissues, which further aggravates insulin resistance and its systemic complications (Decleves et al. 2014; Snel et al. 2012).

Taurine (TAU) is a sulphur-containing amino acid widely studied for its cytoprotective properties in neurons, cardiomyocytes, hepatocytes and kidney cells (Imae et al. 2014). Importantly, TAU is depleted in the plasma and platelets of T2D subjects (De Luca et al. 2001; Franconi et al. 1995). In human subjects and in rats submitted to intravenous lipid infusion, the development of oxidative stress, peripheral insulin resistance and α-cell dysfunction were improved by pre-treatment or coinfusion with TAU (Oprescu et al. 2007; Xiao et al. 2008). Moreover, TAU supplementation in mice fed on a high-fat diet (HFD) improved glucose tolerance and hepatic glucose output, in association with increased phosphorylation and activation of the protein kinase B (Akt), a downstream effector of the insulin signaling cascade (Batista et al. 2013; Ribeiro et al. 2012).

Given the potential of TAU in preventing chronic diseases such as diabetes and hypertension, there is growing interest in its nutraceutical applications. However, only a few long-term human studies with TAU supplementation are available, with most of them lasting less than 6 months (Shao and Hathcock 2008). A study of longer duration in rodents showed that TAU supplementation in streptozotocin-induced diabetic rats for over 2 years reduced blood glucose and increased lifespan (Di Leo et al. 2004). On the other hand, high-fructose fed rats supplemented with TAU for 6 months showed mild improvements in glucose tolerance, but developed fasting hyperglycemia (Larsen et al. 2013). Thus, in order to address the safety of long-term exposure to supraphysiological TAU, in the present study we assessed growth parameters, renal function and glucose homeostasis in mice fed on a HFD and supplemented with TAU for 12 months.

2 Methods

2.1 Experimental Groups and Diets

All experiments were approved by the ethics committee at UNICAMP. Male C57Bl/6 mice were obtained from the breeding colony at UNICAMP and maintained at 21 ± 2 °C, on a 12-h light–dark cycle, with free access to food and water intake. Ten week-old mice were fed either a standard low-fat diet (C) or a high-fat diet (HFD) for 12 months (CH). Half of the CH mice were supplemented with 5 % TAU in their drinking water for the entire experimental period (CHT).

2.2 Assessment of Renal Function

During the last week of intervention, mice were placed individually in metabolic cages and acclimated for 24 h. Afterwards, mice were kept in the cages for 24 h for urine collection in a suitably-designed system that allowed separation from of urine from feces and diet fragments (Tecniplast, Italy). The volume of collected urine was measured and aliquots were kept at −80 °C until analysis. Total urinary proteins and albumin were measured using standard commercial kits, according to the manufacturer's instructions (Laborlab, Brazil).

2.3 Biometric Parameters

At the end of the experimental period, the body weight (BW) was determined. After euthanasia, the liver, kidneys, retroperitoneal and perigonadal fat pads were quickly harvested and weighed.

2.4 Intraperitoneal Glucose Tolerance Test

For the intraperitoneal glucose tolerance test (ipGTT), blood glucose (time 0) was measured using a glucose analyzer (Accu-Chek Performa, Roche Diagnostic, Switzerland) after an overnight fast. A glucose load of 2 g/kg BW was then administered by intraperitoneal injection and blood glucose was measured at 15, 30, 60, and 120 min. Additional blood samples were collected in heparinized tubes at 0 and 30 min of the test, and centrifuged at 10,000 rpm for 5 min at 4 °C. Plasma was then collected and stored at −20 °C for the measurement of plasma insulin by radioimmunoassay—RIA (Ribeiro et al. 2010).

2.5 Intraperitoneal Insulin Tolerance Test

For the intraperitoneal insulin tolerance test (ipITT), blood glucose (time 0) was determined in fed mice. An insulin load of 1.5 U/kg BW was then administered and blood glucose was measured at 10, 15 and 30 min. The slope of the glucose decay curve (kITT) was calculated as previously described (Lehnen et al. 2010).

2.6 Plasma Insulin

Blood samples collected during the ipGTT were centrifuged at 10,000 rpm for 5 min at 4 °C. Plasma was then collected and stored at −20 °C until determination by radioimmunoassay—RIA (Ribeiro et al. 2010).

2.7 Islet Isolation and Static Insulin Secretion

For static insulin secretion, islets were isolated from overnight-fasted mice by collagenase digestion of the pancreas. Groups of five islets were distributed in 24-well plates and pre-incubated for 30 min at 37 °C in Krebs–bicarbonate (KBR) buffer with the following composition: NaCl 115 mmol/L, KCl 5 mmol/L, $CaCl_2$ 2.56 mmol/L, $MgCl_2$ 1 mmol/L, $NaHCO_3$ 10 mmol/L, and HEPES 15 mmol/L, supplemented with 5.6 mmol/L glucose and 0.3 % BSA, and equilibrated with a mixture of 95 % O_2 /5 % CO_2 to give a pH 7.4. This medium was then replaced with fresh buffer and the islets were incubated for a further 1 h with 2.8, 11.1, mmol/L glucose or 30 mmol/L of K^+. At the end of the incubation, the supernatant was collected and stored at −20 °C until insulin measurement by RIA.

2.8 Histological Analysis

Frozen liver and kidney fragments were fixed for 24 h in 4 % formaldehyde/PBS, washed with phosphate buffer solution (PBS) and kept in 70 % ethyl alcohol at 4 °C. Fixed fragments were embedded in paraffin, sectioned and stained with hematoxylin and eosin. Images were captured with a digital camera (Nikon FDX-35, Tokyo, Japan) coupled to a Nikon Eclipse E800 microscope (Tokyo, Japan) under 20× or 40× magnification for liver and kidney slides, respectively.

2.9 Western Blot Analysis

Liver samples were obtained from overnight-fasted mice and immediately homogenized in buffer containing 100 mmol/L Tris pH 7.5, 10 mmol/L sodium pyrophosphate, 100 mmol/L sodium fluoride, 10 mmol/L EDTA, 10 mmol/L sodium vanadate, 2 mmol/L PMSF, and 1 % Triton X-100. The extracts were then centrifuged at 12,000 rpm at 4 °C for 40 min to remove insoluble material. The protein concentration was assayed using the Bradford dye method (Bradford 1976), using BSA as a standard and Bradford reagent (Bio-Agency Lab., São Paulo, SP, BRA). Subsequently, the samples were treated with a Laemmli sample buffer containing β-mercaptoethanol and heated for 5 min at 95 °C before SDS gel electrophoresis. Proteins were then transferred to nitrocellulose membranes and blocked (5 % nonfat dried milk, 10 mmol/L Tris, 150 mmol/L NaCl, and 0.02 % Tween 20) for 1.5 h. The phospho-Akt and Akt ratio was evaluated using a polyclonal antibody against pAkt1/2/3Ser473 (1:1,000, cat. sc-7985R, Santa Cruz Biotechnology) and against Akt1/2/3 (1:1,000, cat. sc-8313, Santa Cruz Biotechnology). Fatty acid synthase (FAS) expression was evaluated using a polyclonal antibody against FAS (1:1,000,

cat. sc-20140, Santa Cruz Biotechnology) and normalized by glyceraldehyde 3-phosphate dehydrogenase protein (GAPDH; 1:1,000, cat. sc-25778, Santa Cruz Biotechnology). Detection of the bands was performed after 1.5 h incubation with a horseradish peroxidase-conjugated secondary antibody (1:10,000, Invitrogen, São 5 Paulo, SP, BRA). The band intensities were quantified by optical densitometry using the free software, Image Tool (http://ddsdx.uthscsa.edu/dig/itdesc.html).

2.10 Statistical Analysis

Results are presented as means + SEM for the number of determinations (n) indicated. The statistical analyses were carried out using a one-way analysis of variance (ANOVA) followed by the Newman–Keuls post hoc test or Student's t test. Significance level was set at $P<0.05$.

3 Results

3.1 Growth Parameters and Adiposity

After 12 months of HFD treatment, mice presented a significant augmentation of BW ($P<0.05$; Fig. 1a) and fat depots ($P<0.05$; Fig. 1b, c). TAU supplementation had no effect upon BW or adiposity. HFD treatment induced hepatomegaly ($P<0.05$) that was enhanced by TAU supplementation ($P<0.01$; Fig. 1d). Histological analysis revealed accumulation of lipid droplets in the liver of CH mice. Consistent with the enhanced HFD-induced liver mass, lipid droplets were more abundant and apparently larger in the CHT mice (Fig. 1e).

3.2 Renal Function

During the last week of intervention, mice were placed in metabolic cages for urine collection. Urinary volume was reduced in all HFD mice without interference by TAU supplementation (C = 1.6 + 0.18; CH = 0.89 + 0.17; CHT = 0.8 + 0.11 mL; $P<0.05$). CHT mice showed a twofold increase in urinary proteins ($P<0.01$; Fig. 2a) and albumin ($P<0.02$; Fig. 2b), in comparison with the C and CH groups. Kidney weight was unaltered by HFD alone, however in association with TAU supplementation it was significantly increased ($P<0.01$; Fig. 2c). Histological analysis of renal cortex from CHT mice revealed the presence of lipid vacuoles in renal tubules (Fig. 2d). Thus, renal dysfunction in long-term TAU-supplemented mice was associated with ectopic lipid accumulation.

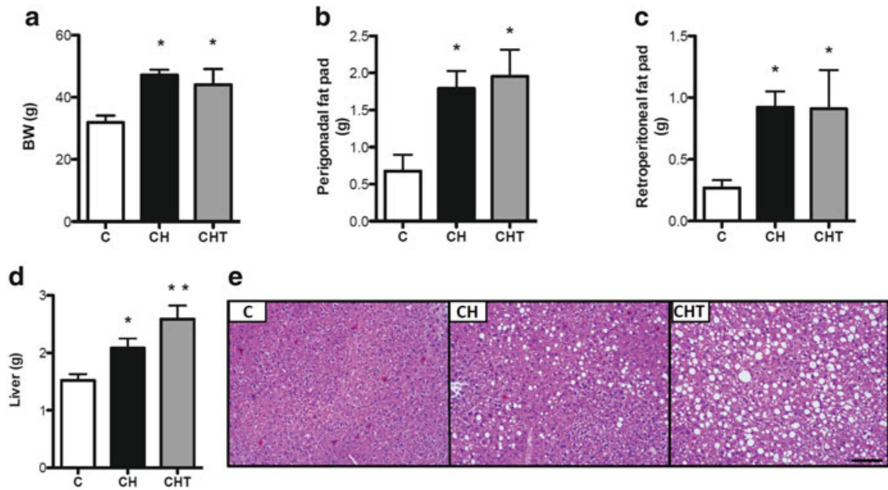

Fig. 1 (**a**) Body weight (**b**) perigonadal and (**c**) retroperitoneal fat pads and (**d**) liver weight of C, CH and CHT mice, n = 4–7. Values are means ± SEM. *P < 0.05 vs. C, One-way ANOVA followed by Newman-Keuls post hoc. (**e**) Light microscopy of HE-stained liver sections from experimental mice. Three livers per group were analyzed. Scale bar 100 μm

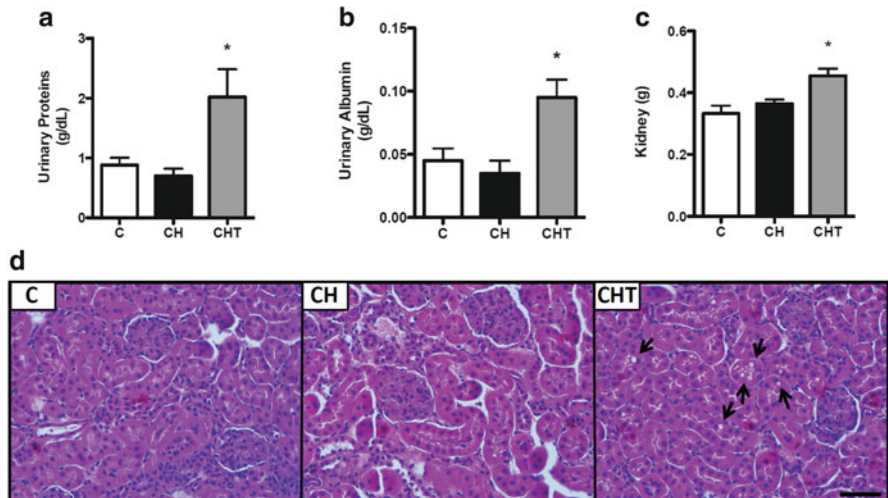

Fig. 2 (**a**) Urinary proteins, (**b**) albumin and (**c**) kidney weight of C, CH and CHT mice, n = 4–7. Values are means ± SEM. *P < 0.05 vs. C, One-way ANOVA followed by Newman-Keuls post hoc. (**d**) Light microscopy of HE-stained kidney cortex sections from experimental mice. Three kidneys per group were analyzed. *Arrows* indicate lipid vacuoles in renal tubules. Scale bar 50 μm

3.3 Glucose Homeostasis

Blood glucose in fed mice was increased in all HFD mice independently of TAU ($P<0.01$; Fig 3a). Strikingly, after a 10 h fast, blood glucose was increased only in TAU-supplemented mice ($P<0.03$). HFD mice were glucose intolerant as assessed by an increased area under glycemic curve (AUC) after a glucose load ($P<0.03$; Fig. 3b, c), which was unaltered by TAU. Prior to the GTT, plasma insulin in fasted mice was similar between all groups (Fig. 3d). In comparison with the fasting state, at 30 min after glucose administration, insulin was increased in C ($P=0.01$) and CHT ($P<0.05$), but not in CH mice (Fig. 3d). Additionally, at 30 min, plasma insulin in CHT mice was significantly higher than C and CH ($P<0.01$).

The results of in vivo insulin secretion were confirmed in isolated pancreatic islets stimulated with glucose (Fig. 4a). At 2.8 and 11.1 mM glucose, insulin secretion from CHT islets was significantly increased ($P<0.01$) compared with C. Furthermore, when stimulated with 30 mM K^+, CH and CHT islets secreted more insulin than C ($P<0.001$). Insulin hypersecretion in glucose intolerant mice suggests insulin resistance. Consistently, CH and CHT mice were insulin intolerant as

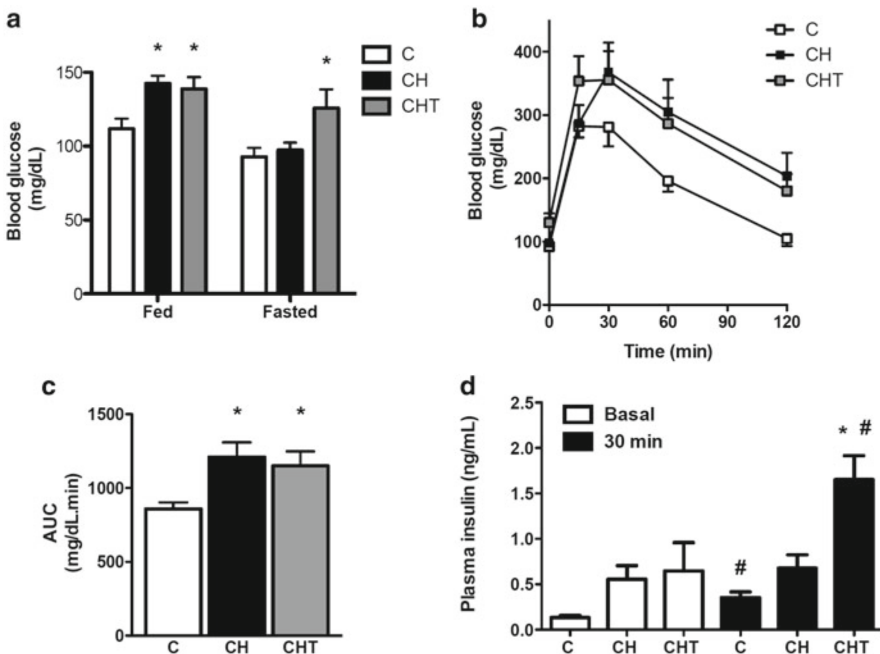

Fig. 3 (**a**) Blood glucose from fed and 10 h-fasted C, CH and CHT mice, n = 6–9. (**b**) Changes in plasma glucose during an ipGTT (2 g/kg). (**c**) Total plasma glucose during the test assessed by the AUC, n = 5–8. (**d**) Plasma insulin during fasting and 30-min after glucose load, n = 4–5. Values are means ± SEM. *$P<0.05$ vs. C, One-way ANOVA followed by Newman-Keuls post hoc; # $P<0.05$ vs. Basal, Student's t test

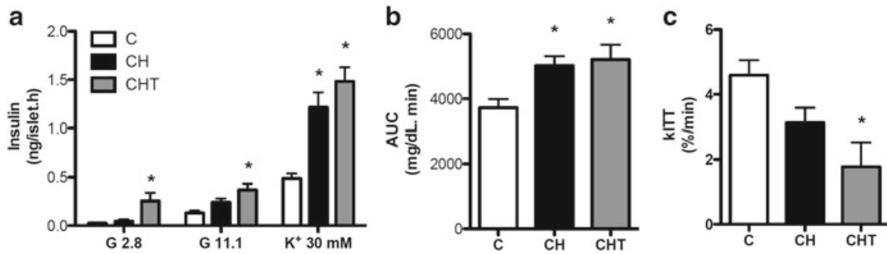

Fig. 4 (**a**) Insulin secretion from isolated islets stimulated with 2.8 and 11.1 mM glucose or 30 mM K^+, n=8. (**b**) Total plasma glucose during the ipITT assessed by the AUC, n=6–9. (**c**) Blood glucose decay constant (kITT) after an ipITT (1.5 U/kg) in C, CH and CHT mice fasted for 2 h, n=4–6. Values are means ± SEM. *P<0.05 vs. C, One-way ANOVA followed by Newman-Keuls post hoc

judged by the increased AUC after an ipITT (P<0.02; Fig. 4b). Analysis of the blood glucose decay constant revealed that the insulin response was severely blunted in CHT mice (P<0.03; Fig 4c).

3.4 Akt Phosphorylation and FAS Expression in Liver

The phosphorylation of Akt is a downstream event of the insulin-signaling cascade, which is critical for the suppression of hepatic glucose output (Kahn et al. 2006). Notably, basal p-Akt/Akt ratio was significantly reduced in the liver of CHT mice (P<0.05; Fig. 5a). Furthermore, expression of the lipogenic enzyme FAS, which catalyzes the formation of long-chain fatty acids from acetyl CoA and malonyl CoA (Wakil 1989), was reduced in the liver of CH mice (P<0.05; Fig. 5b). In CHT mice, hepatic FAS protein content remained similar to C.

4 Discussion

Several studies with rodents and humans have highlighted TAU as a promising therapeutic agent for use in metabolic and cardiovascular diseases (Fujita et al. 1987; Oprescu et al. 2007; Xiao et al. 2008). There are over 30 published clinical trials involving TAU supplementation, which have reported no adverse effects of such an intervention (Shao and Hathcock 2008). However, most interventions lasted less than 6 months, thus making it difficult to address the long-term safety of supraphysiological TAU exposure. In the present study, we aimed to determine the effects of TAU supplementation over 12 months upon growth, lipid accumulation and glucose control in mice fed on a HFD.

Here, BW and fat depots were markedly increased in HFD mice and this was unchanged by TAU supplementation. Available data on the anti-obesity effects of

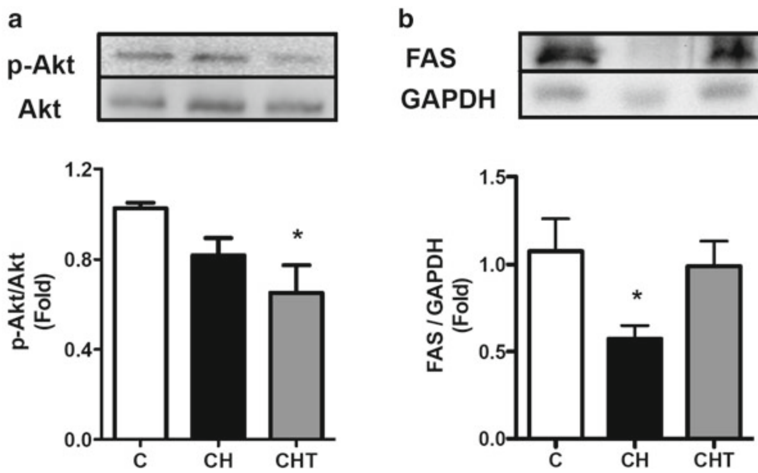

Fig. 5 Western blots for (**a**) p-Akt/Akt and (**b**) FAS/GAPDH in liver fragments from C, CH and CHT mice, n = 4. Values are means ± SEM. *P < 0.05 vs. C, One-way ANOVA followed by Newman-Keuls post hoc

TAU are conflicting and probably influenced by species, strain, dosage, dietary background and duration of supplementation (Nakaya et al. 2000; Nandhini et al. 2005; Ribeiro et al. 2012; Tsuboyama-Kasaoka et al. 2006). Importantly, a recent report from our group, carried out under the same experimental settings (mouse strain, diet and TAU dosage) showed that TAU supplementation for 14 weeks prevented HFD-induced obesity, hypertrophy of adipose tissue and hyperleptinemia (Batista et al. 2013). This suggests that some beneficial effects of TAU supplementation may take place within specific time-windows. Supporting this notion, findings from high-fructose fed rats showed that 2 % TAU supplementation for 30 days prevented hyperglycemia and glucose intolerance (Nandhini et al. 2005), but after 6 months, although a mild improvement in glucose tolerance occurs in TAU-supplemented fructose-fed rats, these rodents presented fasting hyperglycemia (Larsen et al. 2013).

Notably, 12-month TAU supplementation led to enhanced HFD-induced hepatomegaly. Histological analysis showed that the increased organ mass is probably driven by increased lipid accumulation. In contrast with these findings, 2.5 % TAU supplementation for nearly 10 weeks was shown to reduce total fat and triglycerides in the liver of monosodium glutamate-obese rats, but glucose homeostasis remained unaltered (Nardelli et al. 2011). Another study showed that 5 % TAU for 9 weeks to Otsuka Long-Evans Tokushima Fatty (OLETF) rats lowered plasma and hepatic triglycerides and cholesterol (Nakaya et al. 2000). The contrasting ectopic lipid accumulation observed in our study may originate from the redistribution of already synthesized fat, by reduced oxidation of fatty acids or by de novo lipid synthesis. Fat redistribution seems unlikely since the fat depots assessed were unchanged by TAU. It has been previously demonstrated that lipogenesis and the

expression of lipogenic enzymes are reduced in liver and epididymal fat from mice fed on a HFD (Jiang et al. 2009; Ren et al. 2012). Thus, although lipid oxidation was not assessed in the present study, a disruption of the adaptive lipogenic response to the HFD may account for the TAU-induced lipid accumulation since the expression of the hepatic lipogenic enzyme FAS was lowered by the HFD treatment, but kept to the same levels as controls in TAU-supplemented mice. Further studies exploring the regulation of lipogenesis by TAU over extended periods should clarify these contradictory lipid lowering and accumulating properties, attributed to the same molecule.

Long-term TAU supplementation also induced increased kidney weight, in association with renal dysfunction, as indicated by increased urinary proteins and albumin (Ware et al. 2011). Proteinuria and albuminuria suggest loss of the glomerular filtration barrier integrity. Moreover, TAU-supplemented mice showed signs of deranged renal reabsorption as indicated by the development of vacuoles in renal tubules. Although the content of such structures was not investigated, it has been reported that obese mice develop vacuolar structures storing neutral and phospholipids, as well as cholesteryl esters in proximal tubular cells (Decleves et al. 2014; Deji et al. 2009). Whether increased dietary proteins and amino acids are risk factors for kidney disease in healthy subjects is still debated, however increased protein intake may accelerate pre-existing renal disease induced by other factors such as obesity (Knight et al. 2003; Martin et al. 2005). Renoprotective properties were reported with short-term TAU treatments and involved antioxidant and anti-inflammatory mechanisms (Ahmad et al. 2013; Das and Sil 2012); however, as indicated by our results, the presence of persistently high TAU concentrations may interfere with glomerular and tubular processes, leading to dysfunction.

Reports from different research groups have identified TAU as an important regulator of glucose homeostasis. In a HFD mouse model, TAU supplementation improved glucose tolerance, insulin sensitivity and liver Akt phosphorylation (Ribeiro et al. 2012). In rats submitted to lipid infusion-induced insulin resistance, TAU treatment improved insulin resistance by rescuing the phosphorylation status of insulin receptor substrates (IRS) and Akt and by inhibiting the activation of the inflammatory c-Jun NH-terminal kinase 1 (JNK1) in the liver (Wu et al. 2010). The beneficial effects of TAU upon insulin sensitivity and β-cell function have also been reported in human subjects receiving intravenous lipid infusion (Xiao et al. 2008). In contrast with these findings, our data indicate that long-term TAU supplementation induced features of deranged glucose control, such as fasting hyperglycemia, islet hyperfunction and insulin resistance. Impaired hepatic Akt activation in CHT mice is linked with increased FAS expression, in comparison with non-supplemented HFD mice, and the development of a fatty liver. Since these features were absent and even improved after 3.5 months under the same supplementation protocol (Batista et al. 2013; Vettorazzi et al. 2014), we speculate that long-term TAU exposure may elicit additional, and as yet unknown, mechanisms that abrogate its beneficial effects and worsen glucose control.

5 Conclusion

In conclusion, our findings indicate that after long-term exposure, several reported benefits of TAU supplementation are lost. Notably, a 12-month supplementation period at 5 % enhanced the detrimental effects of the HFD upon ectopic lipid accumulation, glucose control and induced features of renal injury. Thus, TAU-based therapies for obese and diabetic subjects should be carefully planned to avoid extending over prolonged periods.

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Taurine Supplementation Enhances Insulin Secretion Without Altering Islet Morphology in Non-obese Diabetic Mice

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Abbreviations

Akt	thymoma viral proto-oncogene/protein kinase B
AUC	Area under curve
BSA	Bovine serum albumin
CHOL	Cholesterol
CTL	Control
$[Ca^{2+}]_i$	Intracellular Ca^{2+} concentration
ERK	Extracellular signal-regulated kinase
Iκ-Bα	Inhibitor of nuclear factor kappa B α
IFN-γ	Interferon γ
IL	Interleukin
ipGTT	Intraperitoneal glucose tolerance test
IGF	Insulin-like growth factor
IR	Insulin receptor
IRS	IR substrates
KRB	Krebs-Ringer bicarbonate
NOD	Non-obese diabetic
NEFA	Non-esterified fatty acids
NFκB	Nuclear factor kappa B
RIA	Radioimmunoassay

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STAT	Signal transducers and activators of transcription
TAU	Taurine
TG	Triglycerides
TNF-α	Tumor necrosis fator α
T1D	Type 1 diabetes
T2D	Type 2 diabetes

1 Introduction

Diabetes mellitus is a multifactorial disease, characterized by chronic hyperglycemia. The two main forms of this disease are type 1 (T1D) and type 2 diabetes (T2D). T1D is typically caused by an autoimmune attack against the β-cells, with a progressive invasion of the pancreatic islets by mononuclear cells, leading to an inflammatory process known as insulitis (Cnop et al. 2005). Several soluble mediators and cytokines released by activated macrophages and T lymphocytes, such as interleukin (IL)-1β, TNF (tumor necrosis factor)-α and IFN (interferon)-γ, cause β-cell dysfunction and death (Thomas and Kay 2000; Eizirik et al. 2008; Ortis et al. 2008).

Non-obese diabetic (NOD) mice spontaneously develop autoimmune diabetes and share many characteristics with human T1D, such as β-cell hyperfunction during the pre-diabetic period with high plasma insulin levels (Amrani et al. 1998), hyperglycemia, presence of autoantibodies and autoreactive T cells (King 2012). As such, NOD mice are often used for the study of T1D mechanisms, genetics and treatments.

Taurine (TAU) is an amino acid that is present at high concentrations in the extracellular and intracellular fluids of mammals. TAU is involved in the regulation of osmolarity (Schaffer et al. 2009), ionic transporter activity (Satoh and Sperelakis 1998; Tricarico et al. 2000), mitochondrial function (Schaffer et al. 2009), glucose homeostasis (Ribeiro et al. 2009; Batista et al. 2013), insulin secretion (Ribeiro et al. 2009, 2010), and presents anti-inflammatory properties (Marcinkiewicz and Kontny 2014). This TAU action may prevent the development of autoimmune diseases, for example, TAU supplementation during gestation and weaning delays the onset of T1D in NOD mice (Arany et al. 2004). Furthermore, TAU treatment during gestation prevents pancreatic islet dysfunction and death in malnourished rats (Boujendar et al. 2003). This amino acid also regulates endocrine pancreatic morphofunction in postnatal life, since TAU administration after weaning prevents islet hypersecretion and hypertrophy in high-fat diet mice (Ribeiro et al. 2012).

In the present study, we evaluated whether TAU supplementation, introduced at birth in NOD mice, modulated pancreatic islet morphology and function in adulthood. In addition, we verified whether the amino acid showed a possible protective action against cytokine-induced β-cell dysfunction.

2 Methods

2.1 Experimental Groups

All experiments were approved by the Ethics Committee in Animal Experimentation, UNICAMP (certificate no.: 1726-1). Female and male newborn NOD mice were distributed into the following groups: NOD mice supplemented (TAU) or not (CTL) with 2 % TAU in drinking water from weaning until 90 days of age. All mice were maintained on a 12 h light/dark cycle (lights on 06:00–18:00 h), controlled temperature (21 ± 2 °C) and air humidity, and allowed free access to food and water.

2.2 Experimental Design for Islet Culture

From weaning until 90 days of age, male *Swiss* mice from the breeding colony, UNICAMP, were maintained on a 12 h light–dark cycle at 21 ± 2 °C, and controlled air humidity. Islets isolated from adult mice were cultured for 12 h in a RPMI-1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 5.6 mM glucose, without (CTL) or with 3 mM TAU (TAU group) plus 10 % fetal bovine serum, 2 % penicillin and streptomycin, and were maintained at 37 °C with an equilibrated mixture of 95 % O_2/5 % CO_2 to maintain pH 7.4. Half of the TAU and CTL cultured islets were also treated with 50 U of recombinant rat IL1-β, or 100 U INF-γ or with the combination of IL-1β plus INF-γ (Invitrogen, Carlsbad, CA, USA).

2.3 Intraperitoneal Glucose Tolerance Test (ipGTT)

For ipGTT, blood glucose levels (time 0) were measured in overnight fasted mice using a glucose analyzer (Accu-Chek Performa, Roche Diagnostic, USA). A glucose load of 2 g/kg body weight was then administered by ip injection and additional blood samples were collected at 15, 30, 60 and 120 min.

2.4 General Nutritional Parameters

At the end of the TAU supplementation, the final body weight and nasoanal length were measured in all groups. In addition, fasted and fed mice were euthanized by decapitation. Retroperitoneal and perigonadal fat pads were collected and weighed. Blood samples were collected and plasma stored at −20 °C. Total plasma protein and plasma albumin were measured using standard commercial kits, according to the manufacturer's instructions (Laborlab, Guarulhos, SP, BRA). Plasma glucose

was also analyzed using a glucose analyzer (Accu-Chek Performa, Roche Diagnostic, USA). Insulin was measured by radioimmunoassay (RIA) using human insulin radiolabeled with ^{125}I (Genesis, São Paulo, SP, BRA) (Ribeiro et al. 2010). Total cholesterol (CHOL), triglycerides (TG) and non-esterified fatty acids (NEFA) were measured using standard commercial kits, according to the manufacturer's instructions (Boehringer Mannhein®, Germany; Merck®, Germany; and Wako®, Germany, respectively).

2.5 TAU Plasma Levels

To measure TAU plasma concentrations in fasted female TAU and CTL NOD mice, 50 μL of plasma were deproteinized by adding 50 μL of 25 % trichloroacetic acid solution and were then centrifuged at 21,000 g for 10 min. Supernatant (25 μL) was collected and homogenized with sample loading buffer (25 μL) (Biochrom 20 reagent kit, Cambridge, UK). An aliquot of 25 μL of the mixture was then resolved by liquid chromatography on a Biochrom 20 plus amino acid analyzer (Amersham Pharmacia, Piscataway, NJ, USA). Amino acid standards were analyzed first, followed by the samples. Amino acids were quantified using Biochrom 20 control software, version 3.05.

2.6 Islet Isolation and Insulin Secretion

Islets were isolated from all mice groups by collagenase digestion of the pancreas. For static incubations, groups of four fresh islets or 12 h-cultured islets were first incubated for 30 min at 37 °C in Krebs-Ringer bicarbonate (KRB) buffer with 5.6 mM glucose and 3 g of BSA/L, and equilibrated with a mixture of 95 % O_2/5 % CO_2 to maintain pH 7.4. This medium was then replaced with fresh buffer and the islets were incubated for 1 h in the presence glucose (see figure legends) or with 2.8 mM glucose plus 30 mM K^+. At the end of the incubation period, aliquots of the supernatant were collected and kept at –20 °C for posterior insulin measurement by RIA. For islet insulin content, groups of four islets were collected and transferred to tubes of 1.5 mL. Deionized water (1 mL) was added to the samples, followed by disruption of the pancreatic cells using a Polytron PT 1200 C homogenizer (Brinkmann Instruments, NY, USA) and the islet insulin content was also measured by RIA.

2.7 Cytoplasmatic Ca^{2+} Oscillations

Fresh pancreatic islets were incubated with 5 μM fura-2 acetoxymethyl ester for 1 h at 37 °C in KRB buffer containing 5.6 mM glucose, 3 g/L BSA, pH 7.4. The islets were then washed with the same medium and were placed in a chamber that was

thermostatically regulated at 37 °C on the stage of an inverted microscope (Nikon UK, Kingston, UK). Islets were then perifused with albumin-free KRB containing 2.8 or 22.2 mM glucose that was continuously gassed with 95 % O_2/5 % CO_2, pH 7.4. A ratio image was acquired every 5 s with an ORCA-100 CCD camera (Hammamatsu Photonics Iberica, Barcelona, Spain), in conjunction with a Lambda-10-CS dual filter wheel (Sutter Instrument Company, CA, USA), which was equipped with 340 and 380 nm, 10 nm bandpass filters, and a range of neutral density filters (Omega opticals, Stanmore, UK). Data were obtained using ImageMaster3 software (Photon Technology International, NJ, USA) (Carneiro et al. 2009).

2.8 Pancreas Morphometry and Immunohistochemistry

For morphometric analysis, pancreases from both groups of mice were removed, weighed and fixed for 16 h in Bouin's solution. After fixation, each pancreas was embedded in paraplast® (Sigma-aldrich Chemicals, St Louis, MO, USA) and exhaustive 5 μm serial sections were obtained (every 20th section) and randomly selected for insulin immunoperoxidase reaction (Inuwa and El Mardi 2005). For imunohistochemistry, paraffin was removed; the sections were rehydrated and washed with 0.05 M tris-saline buffer (TBS) pH 7.4, and incubated with TBS containing 0.3 % H_2O_2 for endogenous peroxidase activity blockade and permeabilized for 1 h with TTBS (0.1 % Tween 20 and 5 g/% of fat free milk in TBS). The sections were incubated overnight with a polyclonal guinea-pig anti-insulin antibody (1:100; Dako North America, Inc., CA, USA) at 4 °C, and after this period, were washed with TBS and again incubated with rabbit anti-guinea-pig IgG conjugated antibody with HRP for 1 h and 30 min. The positive insulin cells were detected with diaminobenzidine (DAB; Sigma-Aldrich Chemicals, St Louis, MO, USA) solution (10 % DAB and 0.2 % H_2O_2 in TBS). Finally, the sections were quickly stained with Ehrlich's hematoxylin and mounted for microscopy observation. All islets present in the sections were covered systematically by capturing images with a digital camera (Nikon FDX-35) coupled to a Nikon Eclipse E800 microscope (Ribeiro et al. 2012). The islet and β-cell areas were analyzed using the free software, Image Tool (http://ddsdx.uthscsa.edu/dig/itdesc.html).

2.9 Western Blotting

To evaluate the ratio between the protein expression of phospho (p)-Akt/Akt and pERK/ERK, islets from TAU and CTL NOD mice were solubilized in homogenization buffer at 4 °C (containing: 100 mM Tris pH 7.5, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM phenylmethylsulfonyl fluoride and 1 % triton-X 100) using a Polytron PTA 20S generator (model PT 10/35; Brinkmann Instruments, Westbury, NY, USA). The extracts were then centrifuged at 12,600 *g* at 4 °C for 30 min to remove insoluble material.

The protein concentration in the supernatants was assayed using the Bradford dye method (Bradford 1976), using BSA as a standard curve and Bradford reagent (Bio-Agency Lab., São Paulo, SP, BRA). For SDS gel electrophoresis and western blot analysis, the samples were homogenized with a loading buffer containing dithiothreitol. After heating at 95 °C for 5 min, the proteins were separated by electrophoresis (40 μg protein/lane, 10 % gels) and were subsequently transferred to nitrocellulose membranes. The membranes were then incubated with specific rabbit polyclonal antibodies to $Akt_{1/2/3}$ (1:1,000, cat. sc-8313), $pAkt_{1/2/3}^{Ser473}$ (1:1,000, cat. sc-7985R), $ERK_{1/2}$ (1:500, cat: sc-94) or pERK1/2 (1:1,000, cat: sc-7383). All primary antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Visualization of specific protein bands was carried out by incubating the membranes with goat anti-rabbit or anti-mouse secondary antibody (1:10,000; Zymed Laboratories, Inc., San Francisco, CA, USA), followed by exposure to X-Ray film. The band intensities were quantified by optical densitometry using the free software, Image Tool (http://ddsdx.uthscsa.edu/dig/itdesc.html).

2.10 Statistic Analysis

Results are presented as means ± SEM for the number of determinations (n) indicated. The statistical analyses were performed using unpaired Student's t-test, employing GraphPad Prism version 5.00 for Windows (GraphPad Software®, San Diego, CA, USA). The level of significance was set at $P<0.05$.

3 Results

3.1 Mice Features

Table 1 shows general biometric and plasma biochemical characteristics in female and male NOD mice supplemented or not with TAU. TAU supplementation did not alter body weight or length, as well as fat depots in both female and male NOD supplemented mice, in comparison with their respective controls (Table 1). In addition, nutritional parameters such as fasting glycemia, plasma insulin, lipids, albumin and total protein levels were similar between the TAU and CTL NOD groups (Table 1).

At 90 days of age, an ipGTT was performed in all mice groups. After glucose administration, glycemia reaches a peak at 15 min and returns to basal glucose levels at 120 min after the test, in both female and male mice groups (Fig. 1a, c). Female TAU-supplemented NOD mice showed lower glycemia at 15 and 30 min for the ipGTT ($P<0.05$ and $P<0.03$; Fig. 1c). Total glycemia during ipGTT, expressed as the area under the glucose curve (AUC), was also reduced in female TAU-supplemented mice when compared with female CTL ($P<0.05$; Fig. 1b).

Table 1 Biometric and biochemical plasma nutritional parameters in fasted 90-day-old female and male NOD mice supplemented or not with 2 % TAU

	CTL	TAU	CTL	TAU
	Female		Male	
Body weight (g)	26±0.6	24±0.4	31±0.8	31±0.4
Nasoanal length (cm)	9±0.2	9±0.1	10±0.3	11±0,2
Retroperitoneal fat pad (mg)	39±6	31±4	141±14	107±13
Perigonadal fat pad (mg)	123±11	92±12	578±45	505±39
Glycemia (mg/dL)	67±5	61±3	66±2	72±4
Insulin (ng/mL)	0.41±0.13	0.45±0.10	0.23±0.04	0.31±0.06
TG (mg/dL)	60±7	74±6	84±10	70±5
NEFA (mM)	1.27±0.09	1.07±0.07	1.01±0.12	1.09±0.10
CHOL (mg/dL)	66±6	65±4	107±12	95±6
Albumin (g/dL)	3.4±0.08	3.4±0.08	3.7±0.07	3.3±0.09
Total proteins (g/dL)	5.07±0.19	5.18±0.19	6.08±0.44	5.55±0.19

Data are means±SEM (n=7–8)

In contrast, no alterations in plasma glucose concentrations during ipGTT were observed between male TAU and CTL NOD mice (Fig. 1c, d). Furthermore, the supplementation methodology applied in our study efficiently increased plasma TAU concentrations, since fasted female supplemented NOD mice presented 28 % higher TAU plasma levels (903±63 μM), when compared with female CTL mice (705±40 μM; $P<0.04$).

3.2 *Pancreatic Islet Function and Morphometry*

Figure 2 shows insulin release in response to glucose or 30 mM K^+. Glucose induced a dose-dependent increase in insulin release in both female and male mice groups (Fig. 2a, c).

TAU enhanced insulin secretion at 22.2 mM glucose in islets isolated from female and male supplemented mice when compared with CTL ($P<0.01$ and $P<0.02$; Fig. 2a, c, respectively). In addition, islets isolated from female TAU groups secreted more insulin in response to 2.8 mM plus 30 mM K^+ ($P<0.05$; Fig. 2b). No modification in insulin secretion in response to this depolarizing agent was observed in male mice groups (Fig. 2d).

Improved islet secretory capacity in response to glucose was linked to a better glucose-induced Ca^{2+} influx in both TAU groups (Fig. 3b, e), in comparison with CTL (Fig. 3a, d), since the total intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) was higher in islets from female and male TAU mice ($P<0.05$ and $P<0.03$; Fig 3c, f).

Table 2 shows pancreatic islet morphometry of all groups of mice. Pancreas weight was similar between TAU and CTL mice of both genders. Histological analysis did not show any alterations in islet, β-cell and non-β-cell areas. In addition, the

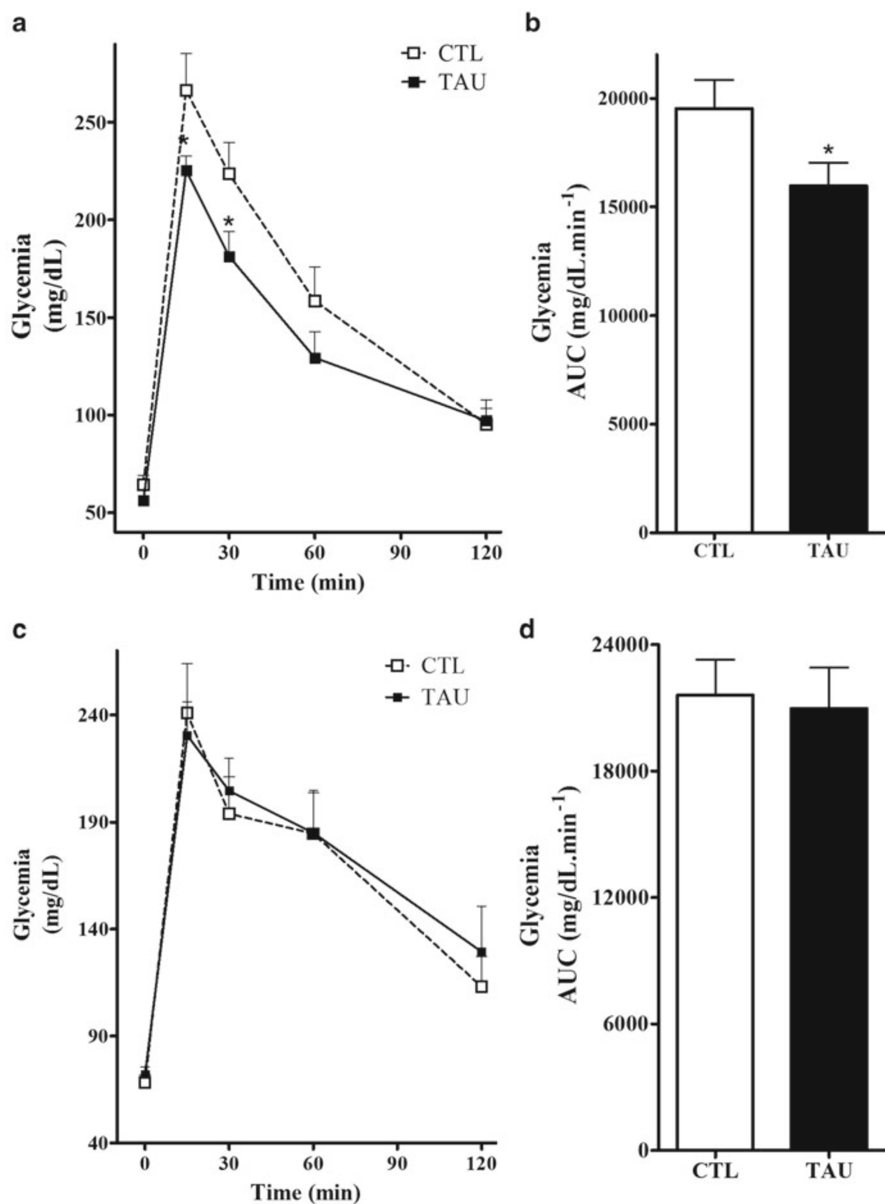

Fig. 1 Changes in plasma glucose levels during ipGTT in 90-day-old TAU-supplemented or CTL NOD female (**a**) and male (**c**) mice. Total plasma glucose concentrations during the ipGTT (**b** and **d**) expressed by AUC. Data are means ± SEM (n = 10–11 mice). *CTL is different from TAU mice in the same gender ($P < 0.05$)

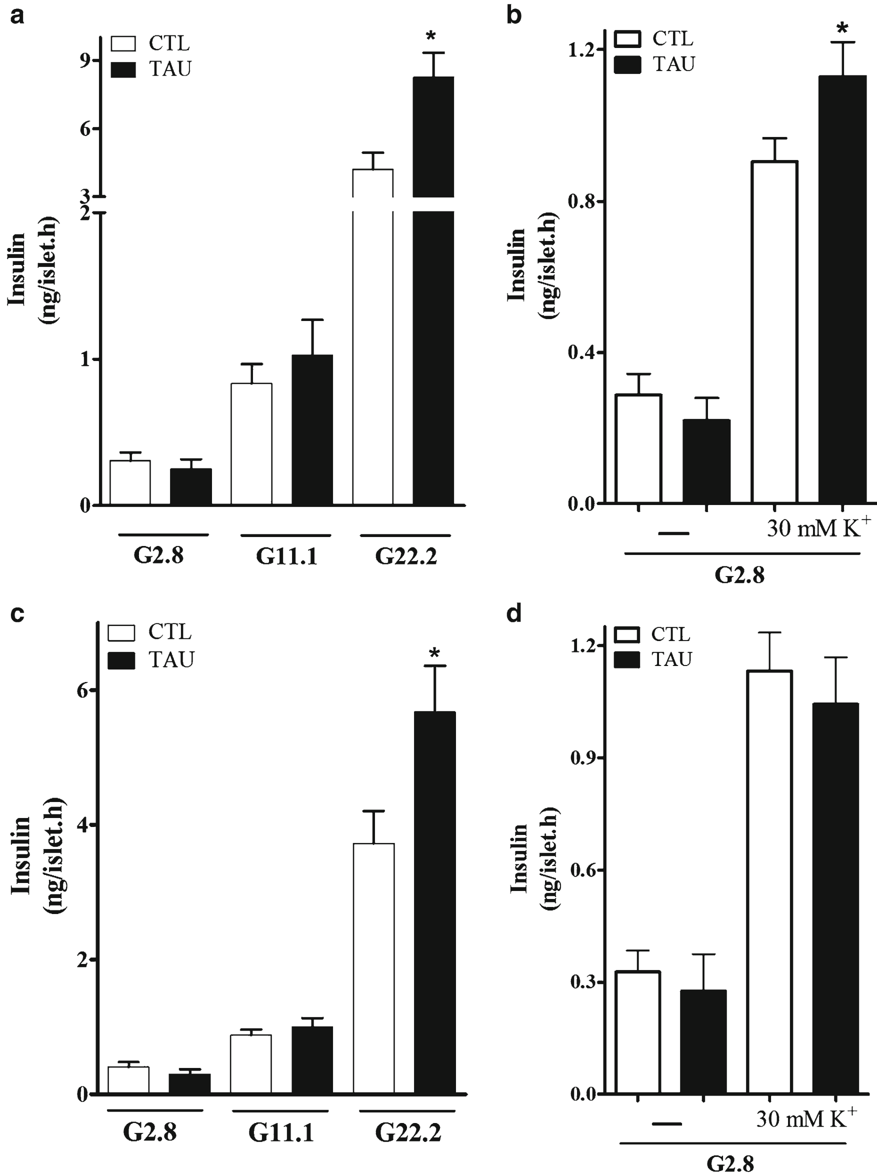

Fig. 2 Insulin secretion in islets isolated from 90-day-old TAU-supplemented and CTL NOD female (**a** and **b**) and male (**c** and **d**) mice. Groups of 4 islets were incubated for 1 h with 2.8, 11.1 or 22.2 mM glucose (G) or with 2.8 mM glucose plus 30 mM K^+. Data are means ± SEM (n = 14–23 groups of islets). *CTL is different from TAU mice under the same experimental conditions ($P < 0.05$)

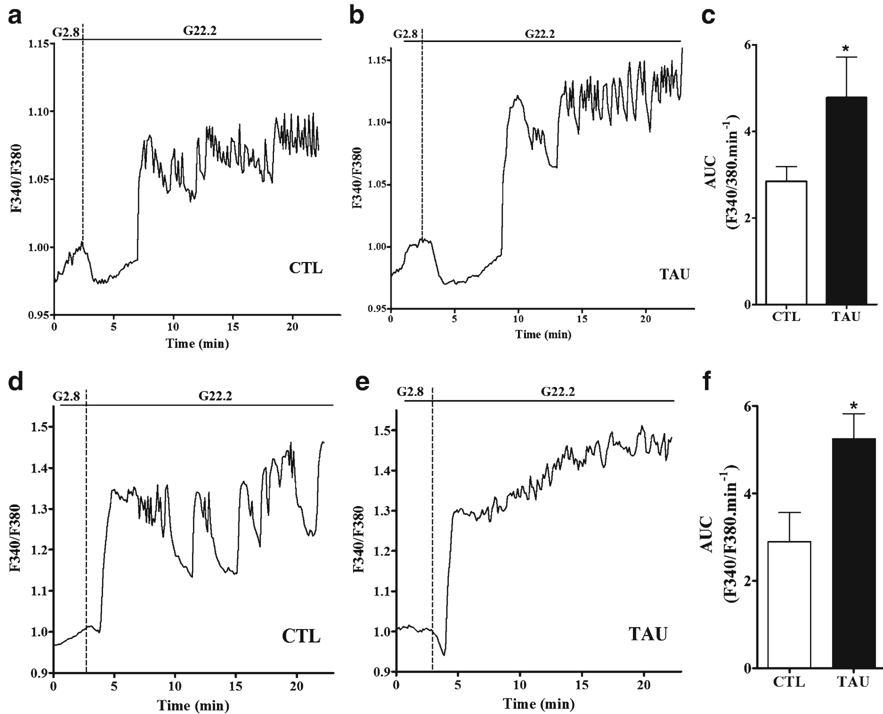

Fig. 3 Representative curves of glucose-induced cytoplasmatic Ca^{2+} oscillations in islets isolated from 90-day-old TAU and CTL NOD female (**a** and **b**) and male (**d** and **e**) mice. The AUC of $[Ca^{2+}]$ i in response to 22.2 mM glucose in female (**c**) and male (**f**) groups. The experiments were performed in a perifusium system in a medium that contained 2.8 or 22.2 mM glucose (G2.8 and G22.2, respectively). Values are the ratio of F340/F380 registered for each group. Data are means ± SEM obtained from 8 to 13 independent experiments. *P < 0.05 vs. CTL

Table 2 Morphometric analysis of pancreases from 90-day-old female and male TAU and CTL NOD mice

	CTL	TAU	CTL	TAU
	Female		Male	
Pancreas weight (mg)	269 ± 14	275 ± 8	294 ± 24	292 ± 11
Islet area (μm²)	5592 ± 565	5438 ± 717	4566 ± 503	4596 ± 518
β-cell area (μm²)	4593 ± 590	4454 ± 660	4122 ± 464	3948 ± 448
Non-β-cell area (μm²)	836 ± 121	707 ± 102	439 ± 53	478 ± 67
β-cell/islet ratio	0.82	0.82	0.90	0.86
Non-β-cell/islet ratio	0.15	0.13	0.10	0.11
Number of islet analyzed	196	151	206	166

Data are means ± SEM (n = 5–7)

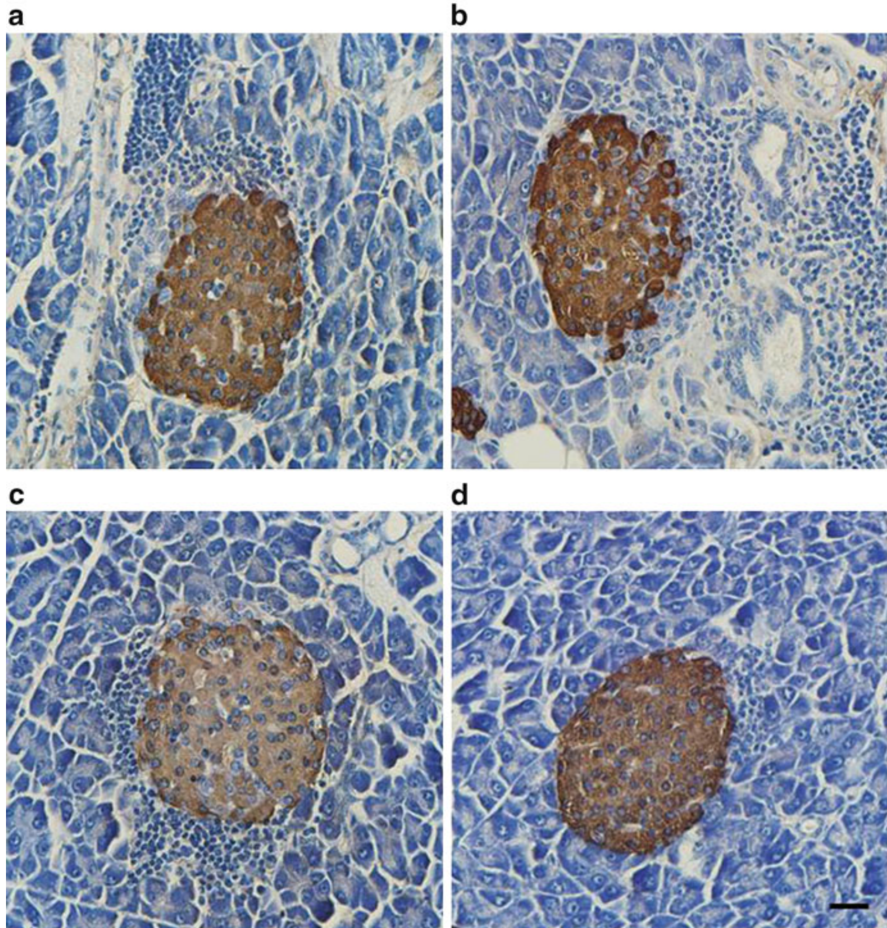

Fig 4 Histological analysis of the endocrine pancreas. Panels show paraplast-embedded pancreas sections (5 μm thick) from 90-day-old TAU and CTL female (**b** and **a**) and male (**c** and **d**) NOD mice, immunolabelled for insulin. Bar: 50 μm

morphology and cytoarchitecture of the islets was not altered by TAU treatment (Fig. 4). Insulin contents in islets from female and male TAU mice (79 ± 10 and 53 ± 14 ng/islet, respectively) were similar to the respective CTL groups (81 ± 7 and 56 ± 8 ng/islet, respectively).

Better islet secretory function in TAU-supplemented NOD mice may be associated with a protective effect of TAU upon cytokine-induced islet dysfunction in NOD islets. Isolated islets from control *Swiss* mice incubated for 12 h in the presence of 50 U IL-1β, or 100 U INF-γ, or with the combination of IL-1β plus INF-γ, showed 49 %, 41 % and 70 % reductions, respectively, in insulin release, when compared with islets incubated without the cytokines ($P<0.01$, $P<0.01$ and $P<0.0001$, respectively). In contrast, when the islets were incubated with 3 mM

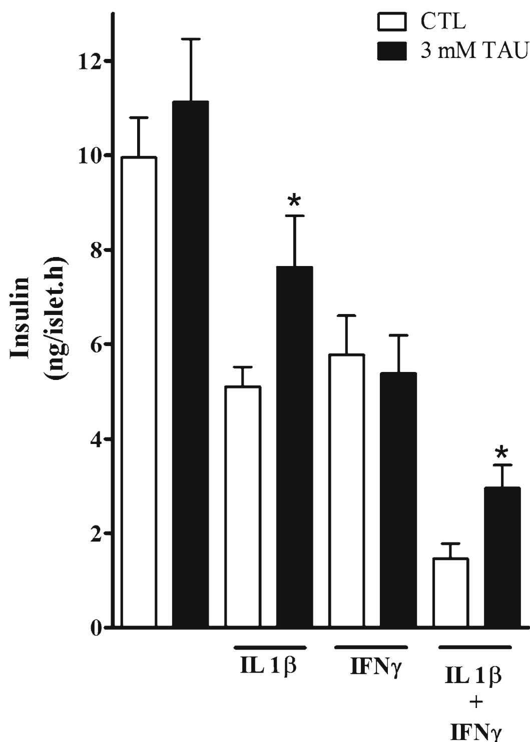

Fig. 5 Insulin secretion in response to 22.2 mM glucose in islets isolated from *Swiss* mice that were incubated for 12 h without or with 50 U IL1-β, 100 U de IFNγ or IL-1β+IFNγ in combination, or not, with 3 mM TAU. Data are means ± SEM (n = 17–22). *P < 0.05 vs. CTL under the same experimental conditions

TAU under the same cytokines conditions, a better insulin secretory function was observed in comparison with CTL islets exposed to IL-1β or IL-1β plus INF-γ ($P<0.05$ and $P<0.02$; Fig. 5). However, TAU did not prevent the reduction in insulin release at 100 U INF-γ.

3.3 Protein Expression

Islets from female TAU mice showed a 1.3- and 1.9-fold increase in pAkt/Akt and $pERK_{1/2}/ERK_{1/2}$ protein expression, respectively, in comparison with the CTL group ($P<0.05$ and $P<0.02$; Fig. 6a, b).

4 Discussion

In this study, we found that TAU supplementation from birth in NOD mice increases glucose tolerance in adult females and, in both genders of NOD mice, improves islet insulin release due to a better $[Ca^{2+}]i$ response to glucose stimulus. These effects were not accompanied by changes in pancreatic islet morphology, but culture experiments showed a potentially protective action of the amino acid against cytokine-induced β-cell secretory dysfunction.

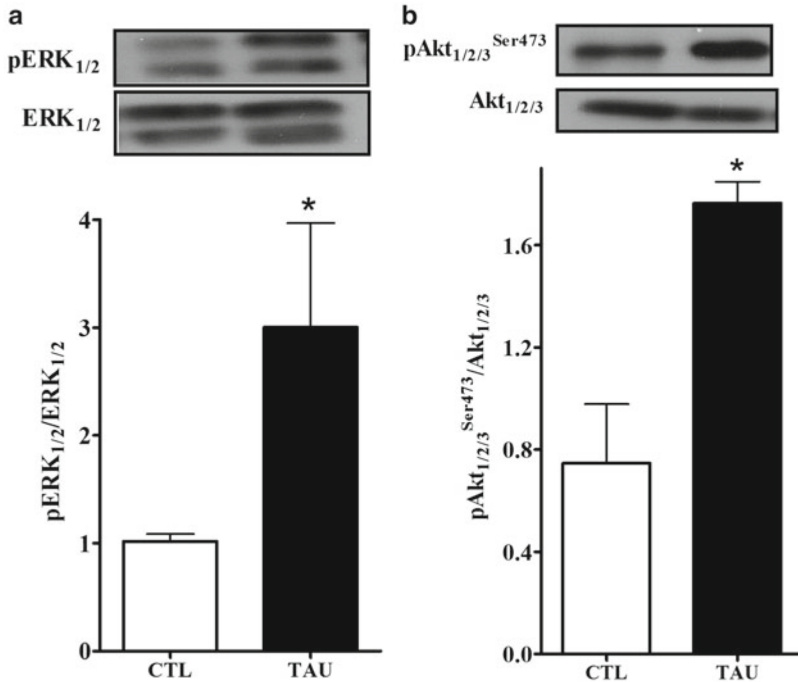

Fig. 6 $pERK_{1/2}/ERK_{1/2}$ (**a**) and $pAkt_{1/2/3}^{Ser473}/Akt_{1/2/3}$ (**a**) protein expressions in isolated islets from female TAU and CTL NOD mice. Bars represent the means ± SEM of the values, determined by optical densitometry (n = 3–5). *P < 0.05 vs. CTL

The better glucose tolerance in female TAU NOD mice may be associated with a possible interaction of TAU with the insulin receptor (IR). Previous reports have demonstrated that an i.p. injection of TAU increases IRβ phosphorylation in a similar way to that stimulated by insulin (Maturo and Kulakowski 1988; Carneiro et al. 2009). Further studies showed that TAU supplementation may restore or improve insulin actions in control and insulin-resistant rodents, leading to a better body nutrient homeostasis (Anuradha and Balakrishnan 1999; Nakaya et al. 2000; Nandhini et al. 2005; Tsuboyama-Kasaoka et al. 2006; Ribeiro et al. 2009, 2012; Batista et al. 2013).

Some evidence points to a β-cell hyperfunction during the pre-diabetic period in T1D, since plasma insulin and pro-insulin levels were higher in subjects (Lo et al. 1992) and experimental rodents with T1D (Nakhooda et al. 1978; Teruya et al. 1993; Amrani et al. 1998). Four-to-six week-old NOD mice presented hyperinsulinemia, lower blood glucose, increased glucose tolerance and insulin secretion, when compared with *C57Bl/6* mice (Amrani et al. 1998). This β-cell hyperfunction is associated with the early lymphocyte invasion, whose autoimmune response onset occurs around the 15th day of life (Trudeau et al. 2000). However no significant islet morphological differences between NOD and CTL mice were observed at 6 weeks of age (Gomez Dumm et al. 1995). Although a gradual reduction in islet mass and secretory function

occurs during the inflammation process, these reductions are associated with high rates of islet-cell proliferation in the pancreases of NOD mice (Sreenan et al. 1999; Kornete et al. 2013). As such, we evaluated islet morphofunction in 12-week-old NOD mice, equivalent to the pre-diabetic stage, to verify the preventive effects of TAU supplementation upon β-cell secretion and $[Ca^{2+}]i$ regulation.

Data have demonstrated a correlation between reduced circulating TAU levels and diabetes development in humans and experimental rodents (Franconi et al. 1995; Anuradha and Balakrishnan 1999; Aerts and Van Assche 2001; Colivicchi et al. 2004; Tsuboyama-Kasaoka et al. 2006). Furthermore, TAU supplementation shows beneficial actions in the prevention of T1D (Arany et al. 2004; Tas et al. 2007) and T2D (Nakaya et al. 2000; Nandhini et al. 2005; Tsuboyama-Kasaoka et al. 2006; Ribeiro et al. 2012; Batista et al. 2013). Our results indicate that TAU supplementation from birth until adulthood does not change islet morphometry, but regulates endocrine pancreatic function with a better glucose-induced insulin secretion, associated with a higher Ca^{2+} influx in islets isolated from female and male TAU groups (Figs. 2 and 3). In addition, female TAU mice presented enhanced insulin secretion upon K^+ stimulus (Fig. 2b).

Previous reports have demonstrated that TAU may regulate β-cell function in metabolic impaired states. Cherif et al. (1998) reported that TAU supplementation during gestation, in dams submitted to protein malnutrition, prevented β-cell dysfunction in their fetuses. This effect was associated with the normalization of β-cell secretory function and the expression of genes involved in β-cell metabolism and proliferation (Cherif et al. 1996, 1998; Reusens et al. 2008). In addition, TAU supplementation during pregnancy in NOD mice delayed the onset of T1D in the offspring (Arany et al. 2004). Our results extend the data available regarding the effect of TAU supplementation upon islet function in T1D, showing that supplementation beginning after birth improves NOD islet function.

TAU improved Ca^{2+} handling, in response to stimulatory glucose concentrations, in islets from NOD-supplemented mice (Fig. 3).We also previously demonstrated that TAU improves nutrient-induced insulin secretion following Ca^{2+} influx in islets isolated from control mice (Ribeiro et al. 2009; 2010). Although we did not verify, in the present study, any change in $[Ca^{2+}]i$ in basal glucose conditions between TAU and CTL islets, different types of experimental diabetic conditions have been shown to lead to altered Ca^{2+} channel content and/or activity in β-cells, contributing to DNA fragmentation and β-cell destruction (Juntti-Berggren et al. 1993; Wang et al. 1996, 1999). Thus, as TAU is reported to be a Ca^{2+} buffering molecule in different cell types (Satoh and Sperelakis 1998; Palmi et al. 1999), we speculate that TAU may protect β-cell function, promoting a better Ca^{2+} handling at basal and stimulatory conditions in T1D.

We did not observe any alteration in islet and β-cell areas when comparing TAU and CTL pancreases (Table 2 and Fig. 4). However, previous data have shown that TAU supplementation during gestation promotes increased DNA synthesis and β-cell mass in NOD mice; this TAU effect was associated with enhanced immunoreactivity to insulin-like growth factor (IGF)-II and a reduction in apoptosis in the NOD pancreas (Arany et al. 2004).

It is known that both IGF-II and insulin can interact with the IR and IGF receptor and that their activation results in phosphorylation of the IR substrates (IRS) (Alvino et al. 2011). Mice that overexpressed IRS-2 in β-cells showed enhanced insulin secretion and did not develop glucose intolerance when fed on a high-fat diet, an effect that was associated with increased phosphorylation of $ERK_{1/2}$ and Akt in β-cells (Hennige et al. 2003). Accordingly, Norquay et al. (2009) demonstrated increased glucose tolerance, islet secretory capacity and β-cell proliferation, in association with a delay in pancreatic lymphocyte infiltration in NOD mice that overexpressed IRS-2 in β-cells. These evidences together with our findings of better glucose tolerance, insulin secretion and increased Akt and $ERK_{1/2}$ activation in islets from female TAU NOD mice (Fig. 6), indicate that TAU may also regulate the IRS-2 pathway in pancreatic islets. The activation of the former mechanism by TAU possibly contributes to the better islet secretory function when control islets were incubated for 12 h with 3 mM TAU, in combination with IL-1β or IL-1β plus INF-γ (Fig. 5).

It should be pointed out that TAU regulates the immune response. TAU reacts with the toxic product of the myeloperoxidase system, hypochlorous acid, forming TAU-chloramines (Marcinkiewicz and Kontny 2014), in turn reducing the production of pro-inflammatory mediators, inflammatory macrophages, neutrophils and lymphocytes and, therefore, controlling the immune response (Marcinkiewicz et al. 1998a, b, 1999). Taking this evidence together with the observation of improved insulin secretion in islets incubated with 3 mM TAU in combination with IL-1β or IL-1β plus INF-γ, we suggest that this amino acid may enhance glucose-induced insulin secretion in NOD mice, protecting β-cells against cytokine-induced dysfunction.

It is known that IL-1β, acting via nuclear factor kappa (NFκ)-B, decreases the first phase of insulin secretion and the expression of several genes involved in β-cells stimulus-secretion coupling (Hostens et al. 1999; Ohara-Imaizumi et al. 2004; Cnop et al. 2005). In contrast, INF-γ interacts with its receptor and activates cytosolic tyrosine kinases, such as janus kinase 1 and 2, which phosphorylate the transcription factor STAT-1; STAT-1 then dimerizes and, once in the nucleus, activates the transcription of diverse genes that lead to β-cell apoptosis (Cnop et al. 2005; Eizirik et al. 2008). Our results suggest that Tau possibly acts via inhibition of the NFκB pathway, and does not have an effective protective action against INF-γ-induced β-cell dysfunction (Fig. 5). Accordingly, TAU supplementation decreases nuclear translocation of NFκB in the heart of alloxan-diabetic rats (Das et al. 2012) and also decreases NFκB activity in *Kupffer* cells from rats with acute pancreatitis (Wei et al. 2012). This action probably occurs at the Iκ-Bα level, since *Jurkat* cells treated with 1 mM TAU chloroamine display a lower TNF-α-induced NFκ-B nuclear translocation due to the oxidation of the methionine 45 residue in the Iκ-Bα protein, a process that decreases its degradation and the release of NFκ-B (Kanayama et al. 2002).

5 Conclusion

In summary, TAU supplementation from birth improved insulin secretion and Ca^{2+} handling at stimulatory glucose concentrations in islets from adult NOD mice. Better islet secretory capacity in TAU groups was not due to modifications in islet

morphometry at 90 days of age, but may be linked to a potential protective effect of TAU upon cytokine-induced islet dysfunction and an increased activation of Akt and $ERK_{1/2}$ in NOD islets.

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Taurine Supplementation Regulates Pancreatic Islet Function in Response to Potentiating Agents in Leptin-Deficient Obese Mice

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Abbreviations

Ach	Acetylcholine
AUC	Area under curve
ANS	Autonomic nervous system
BW	Body weight
BSA	Bovine serum albumin
Cch	Carbachol
C	Control
cAMP	Cyclic adenosine monophosphate
CT	CTL supplemented with Tau
$[Ca^{2+}]i$	Intracellular Ca^{2+} concentration
DAG	Diacylglycerol
DZX	Diazoxide
IP3	Inositol 1,4,5-trisphosphate
KRB	Krebs–Ringer bicarbonate
M3	Muscarinic type 3 receptor
ob	Leptin-deficient obese mice
obT	ob supplemented with Tau
Phe	Phenylephrine
PMA	Phorbol-12-myristate-13-acetate

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PK	Protein kinase
PL	Phospholipase
PNS	Parasympathetic nervous system
RIA	Radioimmunoassay
SNS	Sympathetic nervous system
Tau	Taurine
T2D	Type 2 diabetes

1 Introduction

Obesity is an emerging epidemic problem and predisposes to several chronic non-communicable diseases, such as type 2 diabetes mellitus (T2D). Obesity and T2D are associated with insulin resistance, a condition that leads to a compensatory pancreatic β-cell hyperfunction (Kahn et al. 2006). However, the establishment of T2D is also accompanied by β-cell exhaustion and death (Cerf 2013).

The parasympathetic nervous system (PNS) innervates pancreatic islets and, through acetylcholine (Ach), potentiates glucose-induced insulin secretion. This neurotransmitter interacts with muscarinic type 3 (M3) receptors in β-cells, which activate phospholipase (PL)-C. This enzyme hydrolyses phosphatidylinositol 4,5-bisphosphate and produces inositol 1,4,5-trisphosphate (IP3), which in turn releases Ca^{2+} from intracellular stores; and diacylglycerol (DAG), which activates protein kinase (PK)-C (Boschero et al. 1995; Ahren 2000; Gilon and Henquin 2001). Cholinergic actions may also activate PKA, in turn regulating IP3 receptors and insulin granule exocytosis (Joseph and Ryan 1993; Dolz et al. 2005). Fibers from the sympathetic nervous system (SNS) release norepinephrine, which acts on the β-cell through β-adrenergic or α_2-adrenergic receptors, with consequent increases and decreases in insulin secretion, respectively (Ahren 2000). Several lines of evidence suggest an imbalance in the actions of the autonomic nervous system (ANS) in obesity, with a higher PNS but lower SNS activity (Yoshimatsu et al. 1984; Lee et al. 1989, 1993; Edvell and Lindstrom 1998; Cruciani-Guglielmacci et al. 2005; Shikora et al. 2013). This ANS disruption changes whole body nutrient regulation, leading to more energy storage than expenditure (Scomparin et al. 2009).

Experimental insulin-resistant and/or obese rodents and humans show a reduced SNS action in the endocrine pancreas, which contributes to β-cell hypersecretion (Yoshimatsu et al. 1984; Lee et al. 1989, 1993; Cruciani-Guglielmacci et al. 2005; Balbo et al. 2007; Ahren 2008; Shikora et al. 2013). Pancreatic islets from leptin-deficient obese (ob) mice hypersecrete insulin in response to glucose (Chen et al. 1993) and potentiating agents (Black et al. 1986, 1988; Tassava et al. 1992; Chen and Romsos 1997), and demonstrate a higher proliferation rate that in turn increases the islet-cell mass (Tomita et al. 1992; Edvell and Lindstrom 1998). An imbalance of the ANS may be involved in these endocrine pancreatic compensatory functions in ob mice, since subdiaphragmatic vagotomy reduces insulinemia and islet-cell proliferation (Edvell and Lindstrom 1998).

Taurine (Tau) is a sulfur-containing amino acid that participates in several essential biological processes (Huxtable 1992), such as glucose homeostasis and islet-secretory function regulation (Ribeiro et al. 2009). Tau regulates the M3/PKC and PKA pathways in pancreatic islets (Ribeiro et al. 2010; Batista et al. 2012). In addition, the amino acid modulates ANS function especially in high blood pressure conditions (Fujita and Sato 1988; Sato et al. 1991; Singewald et al. 1997; Hano et al. 2009). However, there is a lack of information about the effects of Tau supplementation on ANS action in pancreatic islets in the obesity. Here, we report that Tau supplementation partially decreases islet hyperfunction in the presence of basal and stimulatory glucose concentrations in male ob mice, an effect that is associated with a reduction in hormone secretion when PKC and PKA are activated. In female ob mice, Tau treatment normalized insulin secretion at basal glucose concentrations and also preserved the normal islet secretory function in response to the cholinergic/PKC and SNS pathways.

2 Methods

2.1 Experimental Groups

All experimental procedures were developed in accordance with the Ethics Committee in Animal Experimentation, UNICAMP (certificate no.: 2018-1). From weaning until 90 days of age, male and female *C57Bl/6* (C group) or *ob/ob* (ob) mice were distributed into four groups: C; C that received 5 % Tau in their drinking water (CT); ob or obT. All mice groups were maintained on a 12 h light–dark cycle (lights 8:00–20:00 h), with controlled humidity and temperature (21 ± 2 °C), with free access to standard laboratory rodent chow (Nutrilab, Colombo, PR, BRA) and water.

2.2 Evaluation of Obesity

At 90 days of age, the final body weight (BW) and nasoanal length were measured in all mice groups for calculation of the Lee index [from the ratio of BW (g)$^{1/3}$/nasoanal length (cm).1000] (Bernardis and Patterson 1968). Subsequently, all mice groups were euthanized and retroperitoneal and perigonadal fat pads were collected and weighted.

2.3 Islet Isolation and Insulin Secretion

Islets were isolated by collagenase digestion of the pancreas. For insulin secretion, groups of four islets were incubated for 30 min at 37 °C in Krebs–Ringer bicarbonate (KRB) buffer containing 115 mM NaCl, 5 mM KCl, 10

mM $NaHCO_3$, 2.56 mM $CaCl_2$, 1 mM $MgCl_2$ and 15 mM HEPES (Sigma-Aldrich Chemical, St Louis, MO, USA), supplemented with 5.6 mM glucose plus 0.3 % of BSA, and equilibrated with a mixture of 95 % O_2/5 % CO_2 to give pH 7.4. This medium was then replaced with fresh KRB buffer and the islets were further incubated for 1 h with 5.6 or 11.1 mM glucose alone or in combination with 10 μM forskolin, 100 nM phorbol 12-myristate 13-acetate (PMA), 100 μM carbachol (Cch) or 10 μM phenylephrine (Phe; Sigma-Aldrich Chemical, St Louis, MO, USA). At the end of the incubation period, the insulin content of the medium was measured by RIA using radiolabeled ^{125}I human insulin (Genesis, São Paulo, SP, BRA), as previously reported (Ribeiro et al. 2010).

2.4 Recording of Cytoplasmic Ca^{2+} Mobilization

Fresh pancreatic islets were incubated for 1 h in a KRB buffer containing 5.6 mM glucose, 0.3 % BSA and gassed with a mixture of 95 % O_2/5 % CO_2 to give pH 7.4. Afterwards, the islets were incubated under the same described above conditions for a further hour with 5 μM fura-2-acetoxymethyl ester, a Ca^{2+}-sensitive dye (Calbiochem, San Diego, CA, USA). Subsequently, single islets were placed in a chamber that was thermostatically regulated at 37 °C, over poly L-lysine-treated glass coverslips on the stage of an inverted epifluorescence microscope (Nikon Eclipse TE200, Tokyo, Japan). Islets were perifused with a Ca^{2+}-free KRB containing 11.1 mM glucose, 250 mM diazoxide (DZX) and 10 mM EGTA with or without 100 μM Cch. A ratio image was acquired approximately every 3 s with a Cool One camera (Photon Technology International, NJ, USA) using a dual filter wheel equipped with 340 nm, 380 nm and 10 nm bandpass filters, and a range of neutral density filters (Photon Technology International, NJ, USA). Data were acquired using the Image Master version 5.0 (Photon Technology International, NJ, USA) (Carneiro et al. 2009).

2.5 Statistic Analysis

Results are presented as means ± SEM for the number of experiments indicated. Statistical analyses were carried out using two-way analysis of variance (ANOVA) followed by the Holm-Sidak post test and performed using SigmaStat version 3.5 for Windows (Jandel Scientific Software Inc., San Jose, CA, USA) and the level of significance was set at $P < 0.05$.

3 Results

3.1 *Obesity Evaluation*

Table 1 shows general obesity parameters evaluated at 90 days of age in ob and C mice supplemented, or not, with Tau. Increases of 126 % and 153 % in BW were observed in male and female ob mice, respectively, in comparison with their C groups ($P<0.0001$). Male and female ob mice presented longer body lengths (9.2±0.3 and 9.5±0.3 cm, respectively) than C mice (8.4±0.1 and 8.3±0.3 cm; $P<0.03$ and $P<0.01$, respectively). Both, male and female ob groups presented a 21 % higher Lee index, when compared with C mice ($P<0.0001$). The retroperitoneal and perigonadal fat stores were 8.7 and 5.3-fold higher in male ob, and presented a 3.7 and 8.6-fold increase in the female ob group, in comparison with their respective C groups ($P<0.0001$; Table 1). Tau supplementation promoted a reduction of 8 % in the Lee index in the male obT group when compared with ob mice ($P<0.03$), without altering other obesity parameters (Table 1) and body length in male and female obT mice (9.6±0.2 and 9.3±0.3 cm, respectively).

3.2 *Insulin Secretion in Response to Glucose*

Figure 1 shows insulin secretion in response to 5.6 and 11.1 mM glucose in islets isolated from ob and C mice supplemented or not with Tau. Male and female ob groups secreted high amounts of insulin at basal glucose concentrations ($P<0.0001$; Fig. 1a, b), but only islets isolated from male ob mice hypersecreted insulin at stimulatory glucose conditions, when compared with C mice ($P<0.0001$; Fig. 1b). Tau supplementation prevented islet hypersecretion in response to glucose, since in male and female obT groups, insulin release at 5.6 mM glucose was 32 % and 40 %,

Table 1 Obesity parameters evaluated in 90-day-old male and female ob and C mice supplemented, or not with 5 % Tau

		C	CT	ob	obT
BW (g)	Male	23±1[a]	22±1[a]	52±1[b]	49±2[b]
	Female	19±1[a]	18±1[a]	48±2[b]	48±2[b]
Lee Index	Male	339±4[a]	325±9[a]	409±12[b]	374±9[c]
	Female	321±9[a]	332±14[a]	387±9[b]	393±13[b]
Retroperitoneal fat pad (mg/g BW)	Male	3.4±0.5[a]	2.5±0.5[a]	33.0±3.9[b]	26.9±4.2[b]
	Female	5.2±1.5[a]	3.6±0.7[a]	24.3±8.5[b]	25.6±4.1[b]
Perigonadal fat pad (mg/g BW)	Male	9.6±1.2[a]	7.6±0.2[a]	60.2±3.6[b]	65.1±15.3[b]
	Female	10.2±2.0[a]	7.0±1.5[a]	97.4±4.3[b]	87.6±9.9[b]

Data are means±SEM (n=4–5 mice). Different letters represent significant differences (Two-way ANOVA followed by Holm-Sidak post test, $P<0.05$)

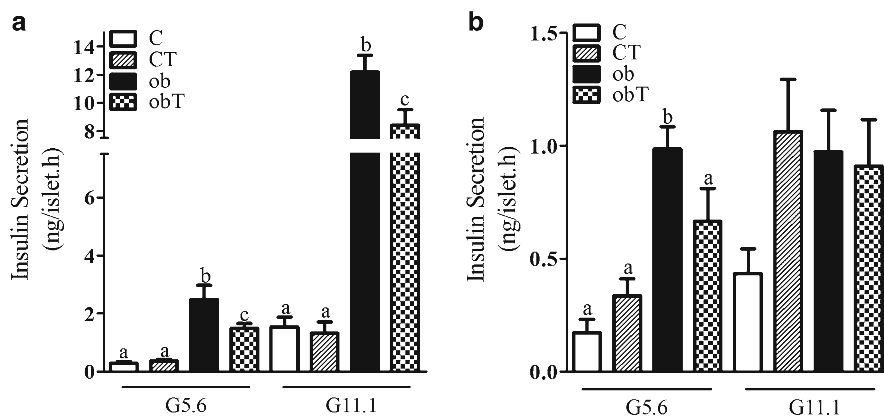

Fig. 1 Glucose-induced insulin secretion in islets from 90-day-old male (**a**) and female (**b**) ob and C mice supplemented, or not, with Tau. Groups of 4 islets were incubated for 1 h with different glucose (G) concentrations, as indicated. Each bar represents mean ± SEM (n = 6–15). Different *letters* represent significant differences between the groups in the presence of the same glucose concentrations (Two-way ANOVA followed by Holm-Sidak post test, $P < 0.05$)

respectively, which was lower than of that observed for ob islets ($P < 0.01$ and $P < 0.03$). In addition, at 11.1 mM glucose, Tau treatment prevented insulin hypersecretion in islets from male obT mice with a 31 % reduction in comparison with ob islets ($P < 0.009$; Fig. 1a).

3.3 Insulin Secretion and Intracellular Ca^{2+} Mobilization in Response to Cholinergic/PKC Pathway Activation

The incubation of isolated islets with 5.6 and 11.1 mM glucose, in combination with 100 µM Cch, a M3 receptor agonist (Yoshimura et al. 2006), leads to a higher increment in insulin release at basal and stimulatory glucose concentrations in islets from male ob mice ($P < 0.0001$; Fig. 2a). However, in the female ob group, Cch enhanced insulin secretion only at 11.1 mM glucose ($P < 0.0001$; Fig. 2b). The cholinergic agonist also increases insulin release at 5.6 mM glucose in the male and female C group ($P < 0.002$ and $P < 0.04$), and at 11.1 mM glucose in both C and CT islets ($P < 0.05$). Tau supplementation prevented Cch-induced hypersecretion only in the obT female groups (Fig. 2b).

When insulin release was investigated in the presence of 100 nM PMA, which activates PKC (Gilon and Henquin 2001), both male and female ob groups presented an increase in insulin secretion at 5.6 and 11.1 mM glucose, showing a higher increment in the insulin release when compared with C islets ($P < 0.0001$; Fig. 2c, d). PMA also increased insulin release under basal and stimulatory glucose conditions in C and CT islets ($P < 0.05$). Tau supplementation normalized the insulin release,

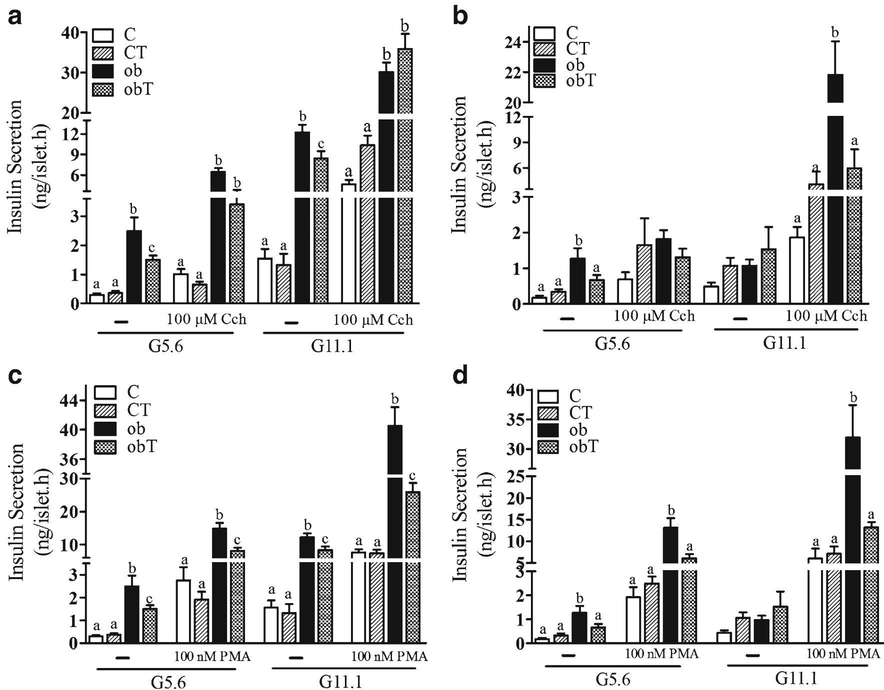

Fig. 2 Insulin secretion induced by Cch (100 μM) or PMA (100 nM) in islets from male (**a** and **c**) and female (**b** and **d**) ob and C mice, supplemented or not with Tau. Islets were incubated for 1 h at 5.6 or 11.1 mM glucose (G), with or without Cch or PMA. Each bar represents mean ± SEM (n = 6–16). Different *letters* indicate significant differences between the groups in the same condition evaluated (Two-way ANOVA followed by Holm-Sidak post test, $P < 0.05$)

under PMA stimulus, in islets from female obT mice (Fig. 2d); in the male obT group, reductions of 40 % and 36 % in insulin secretion at 5.6 and 11.1 mM glucose plus PMA, respectively, were observed, in comparison with the male ob group ($P < 0.0001$; Fig. 1c).

We also analyzed Ca^{2+} mobilization from internal stores in islets isolated from ob and C mice supplemented, or not, with Tau. For this, 100 μM Cch was added to a perifusion system with a Ca^{2+}-free medium, containing 11.1 mM glucose, 250 μM DZX, and 10 mM EGTA. The Cch-induced increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) was higher in islets from male and female ob groups, in comparison with C islets (Figs. 3a, d and 4a, d, respectively). The areas under the curves (AUC) in islets from female ob mice (Fig. 4c) and the amplitude of the $[Ca^{2+}]i$ (Figs. 3f and 4f) in response to Cch, in ob islets from both genders, were higher in comparison with C islets ($P < 0.04$, $P < 0.001$ and $P < 0.0002$, respectively). The normalization of the Cch-induced insulin secretion in the female obT group was associated with a partial reduction in the total intracellular Ca^{2+} mobilization (Fig. 4c), without alterations in the amplitude of $[Ca^{2+}]i$ (Figs. 3f and 4f).

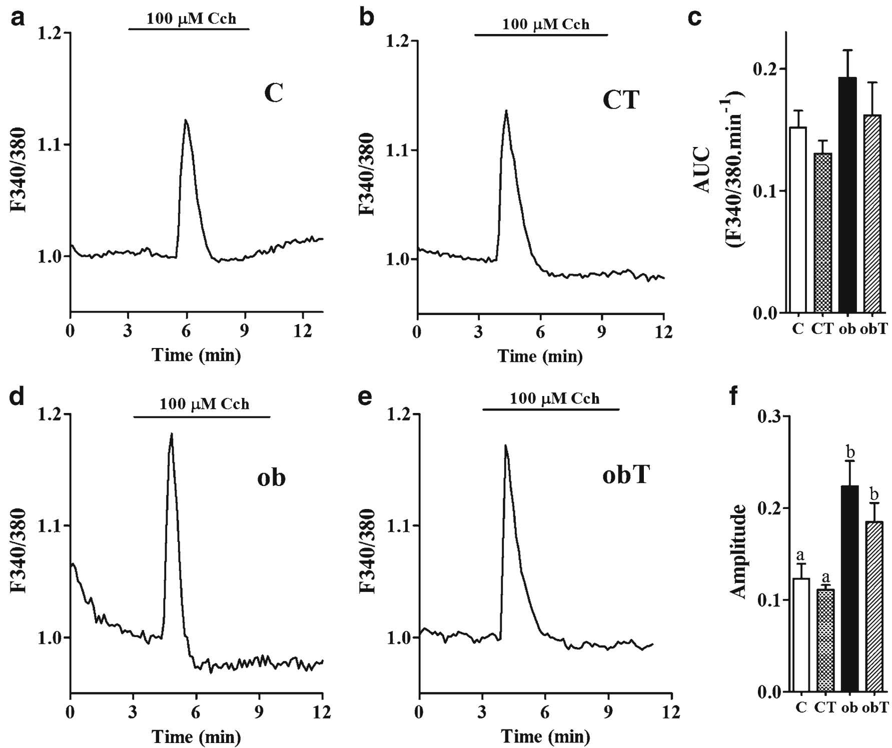

Fig. 3 Cch (100 μM) induced Ca^{2+} mobilization from intracellular stores in islets isolated from male C (**a**), CT (**b**), ob (**d**) and obT (**e**) mice. The experiments were performed in a perifusion system in a Ca2+-free medium containing: 11.1 mM glucose (G11.1), 250 μM DZX, and 10 mM EGTA. Values are the ratio of F340/F380 registered for each group. Data are means ± SEM of AUC (**c**) and amplitude (**f**) of $[Ca^{2+}]i$ obtained from 8 to 10 independent perifusion experiments. Different *letters* represent significant differences (Two-way ANOVA followed by Holm-Sidak post test, $P<0.05$)

3.3.1 Forskolin and Phe Effects on Insulin Secretion

Figures 5a, b show insulin secretion in response to 10 μM forskolin, which activates adenylyl cyclase and increases cytosolic cAMP (cyclic adenosine monophosphate) levels (Dyachok et al. 2008). Forskolin induced a higher increase in insulin secretion, under basal and stimulatory glucose conditions, in all ob groups in comparison with C islets ($P<0.0001$). In addition, forskolin increased insulin release at 5.6 mM glucose in the male C group ($P<0.0001$), and in C and CT islets at stimulatory glucose concentrations ($P<0.05$). Tau supplementation decreased insulin release in response to forskolin in male obT at 5.6 and 11.1 mM glucose, when compared with ob islets ($P<0.0001$ and $P<0.007$; Fig. 5a). No alteration in forskolin-induced secretion was observed between female obT and ob mice (Fig. 5b).

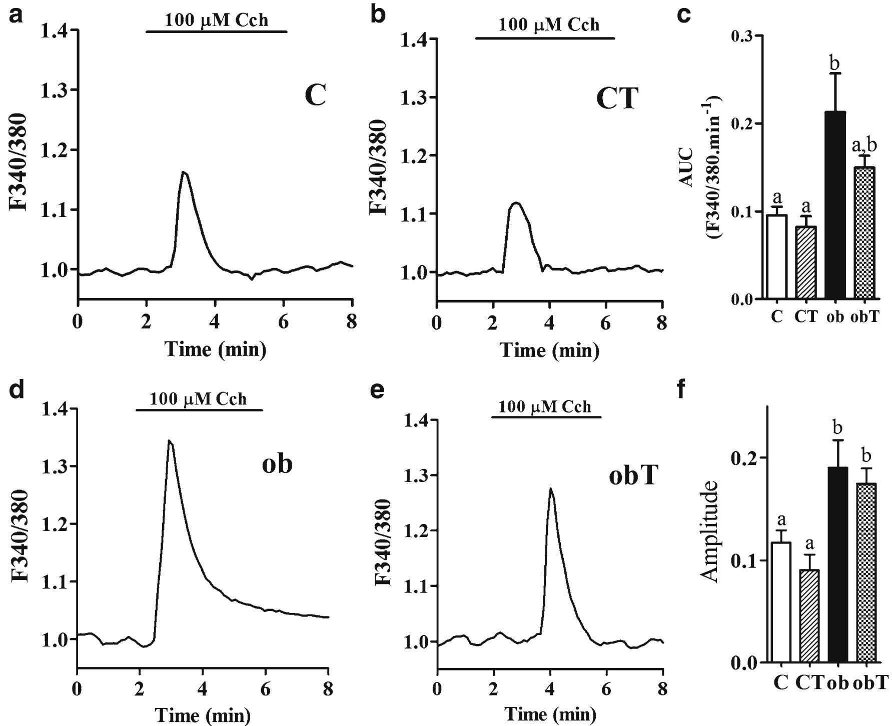

Fig. 4 Cch (100 μM) induced internal Ca^{2+} mobilization in islets isolated from female C (**a**), CT (**b**), ob (**d**) and obT (**e**) mice. The experiments were performed in a perifusion system in a Ca^{2+}-free medium containing: 11.1 mM glucose (G11.1), 250 μM DZX, and 10 mM EGTA. Values are the ratio of F340/F380 registered for each group. Data are means ± SEM of AUC (**c**) and amplitude (**f**) of [Ca2+]i obtained from 6 to 7 independent perifusion experiments. Different *letters* represent significant differences (Two-way ANOVA followed by Holm-Sidak post test, $P<0.05$)

In the next series of experiments, we analyzed the inhibition of insulin release in all groups of islets in the presence of 10 μM Phe, a α-adrenergic agonist (Hillaire-Buys et al. 1985; Freitag et al. 1998). The male ob group exhibited reductions of 46 % and 76 % in insulin secretion at 5.6 and 11.1 mM glucose, respectively, in response to the adrenergic agonist ($P<0.04$ and $P<0.0001$; Fig. 5c). However, no significant decrease in insulin secretion in islets isolated from female ob mice, at 5.6 and 11.1 mM glucose plus Phe, was observed (Fig. 5d). Tau treatment showed a dual effect in response to Phe, with a normalization of Phe-induced inhibition in insulin release at 5.6 and 11.1 mM glucose in the female obT group (Fig. 5d), but no alteration in the adrenergic agonist action in islets from male obT mice was observed when compared with ob group (Fig. 5c).

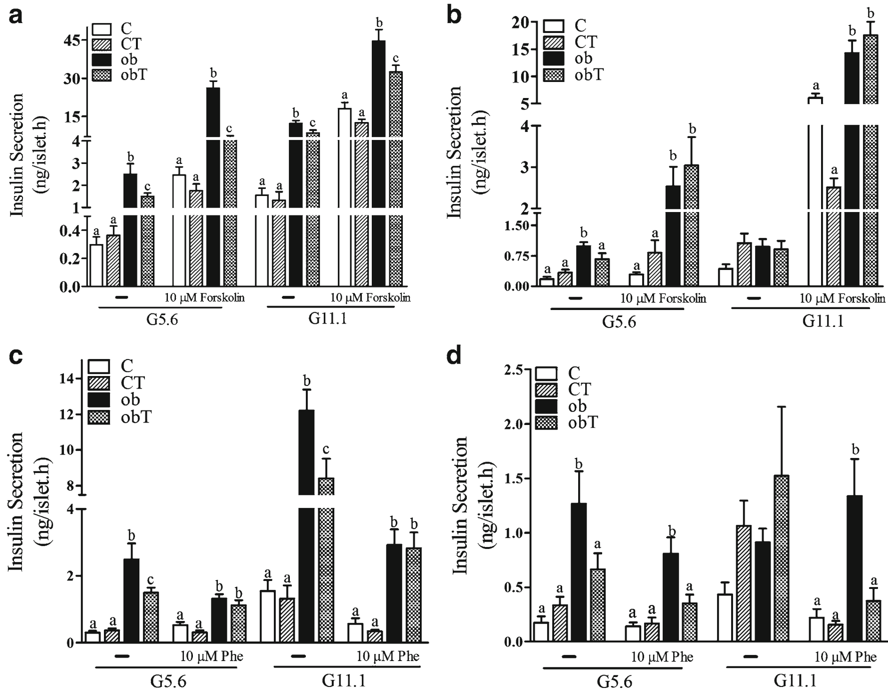

Fig. 5 Insulin secretion in the presence of 10 μM forskolin or 10 μM Phe in islets isolated from male (**a** and **c**) and female (**b** and **d**) ob and C mice supplemented or not with Tau. Islets were incubated for 1 h at 5.6 or 11.1 mM glucose (G), with or without forskolin or Phe. Data are means ± SEM (n = 6–16). Different *letters* represent significant differences (Two-way ANOVA followed by Holm-Sidak post test, $P < 0.05$)

4 Discussion

Our results demonstrate that pancreatic islets from ob mice hypersecrete insulin in response to glucose. The higher insulin secretion observed under basal glucose conditions is a compensatory secretory function in response to the severe insulin resistance noticed in these rodents (Tomita et al. 1992). Here, we verified that ANS is involved in the former mechanism, since ob islets isolated from both genders of mice presented a greater increase in insulin release following the activation of M3/PKC and PKA pathways, whereas the inhibition of the insulin secretion in response to α-adrenergic activation was impaired in female ob mice. Tau supplementation decreased glucose-induced insulin secretion in islets from male obT mice in an effect that was associated with lower PKC and PKA–induced insulin release. However, in the female obT group, Tau normalized insulin secretion, under basal glucose levels, in response to the M3/PKC pathway and following inhibitory adrenergic action.

As expected, ob mice of both genders presented a morbid obesity condition (Lindstrom 2010), with enhanced BW, Lee index and body fat depots (Table 1).

While several mechanisms have been suggested to be involved in the manifestation of obesity and its maintenance, an imbalance of the action of ANS with a higher PNS, but lower SNS action, has been reported in several experimental models and humans with this syndrome (Yoshimatsu et al. 1984; Lee et al. 1989, 1993; Edvell and Lindstrom 1998; Cruciani-Guglielmacci et al. 2005; Shikora et al. 2013).

The higher PNS and lower SNS action in obesity and insulin-resistant states leads to insulin hypersecretion and increased islet-cell proliferation (Lee et al. 1989, 1993; Edvell and Lindstrom 1998; Ahren 2008). Here, we also confirm that pancreatic islets from ob mice showed a lower threshold for glucose-induced insulin secretion (Chen et al. 1993), since ob islets from both gender secreted high amounts of insulin in response to 5.6 mM glucose (Fig. 1). In addition, we also found that islets from ob mice hypersecreted insulin in response to the activation of the M3/PKC pathway (Fig. 2), an effect that was accompanied by an enhanced Ca^{2+} mobilization from internal stores in the ob group (Figs. 3 and 4). Insulin hypersecretion in response to Ach and PKC activation have been previously reported in ob islets (Tassava et al. 1992; Chen and Romsos 1997). In these studies, Ach-induced insulin hypersecretion was associated with PKC activation, since PKC downregulation restored insulin secretion in ob islets to similar levels to those observed in lean mice (Chen and Romsos 1997). Our results extend the data regarding ob islet function, since a higher intracellular Ca^{2+} mobilization was noted (Figs. 3 and 4), indicating that the higher $[Ca^{2+}]i$, together with PKC, may enhance the efficiency of granule exocytosis in the ob group.

The amino acid, Tau, improves glucose homeostasis and pancreatic islet function in response to nutrients and agents that potentiate insulin release (Ribeiro et al. 2009; Ribeiro et al. 2010; Batista et al. 2012). Tau also prevents islet hypersecretion and hypertrophy in obese mice fed on a high-fat diet (Ribeiro et al. 2012). In our study, Tau supplementation decreased insulin secretion following a PMA stimulus in islets from obT mice (Fig. 2a, c), and normalized insulin secretion upon Cch and PMA stimulation, but partially decreased Ca^{2+} internal flux in the female obT group (Fig. 2b, d). Although a direct action of Tau in PNS activity has not been reported, several studies have demonstrated that Tau may regulate cholinergic action and PKC activation under different experimental conditions. Tau dose-dependently decreased skeletal muscle contraction in response to Cch (Lehmann and Hamberger 1984). This amino acid decreases the activation of conventional and atypical PKC (Wang et al. 2009; Das et al. 2010), but also has protective effects on cardiomyocytes via the activation of PKCε (Ito et al. 2009). In islets isolated from Tau-supplemented mice, no alteration in PKC-induced insulin secretion was observed (Ribeiro et al. 2010). However, normalization of M3 receptor protein content and Cch-induced insulin release was seen in islets from malnourished rats supplemented with Tau (Batista et al. 2012). These data demonstrate that Tau may regulate the cholinergic/PKC pathway and, in obesity, its action decreased islet hyperfunction.

In pancreatic islets from *Goto-Kakizaki* diabetic rats, Ach improved glucose-induced insulin secretion via the activation of the PKA pathway (Dolz et al. 2005). Here, we confirm that islets from male and female ob mice also hypersecreted insulin in response to forskolin under basal and stimulatory glucose concentrations

(Fig. 5a, b) (Black et al. 1986, 1988). Additionally, we showed that only islets from male ob mice respond to the inhibitory action of Phe (Fig. 5c), while an efficient decrease in insulin release in response to norepinephrine in 8-week old female ob mice has been reported (Tassava et al. 1992). Our results are in accordance with several studies that have demonstrated an impaired SNS inhibitory action in β-cells in genetically- or diet-induced obese rodents, which contributes to insulin hypersecretion (Lee et al. 1989, 1993; Cruciani-Guglielmacci et al. 2005; Ribeiro et al. 2014). Furthermore, insulin-resistant conditions lead to decreased SNS action in humans, since increased insulinemia, circulating C-peptide levels and reductions in norepinephrine plasma levels have been observed in healthy subjects submitted to 48 h triglyceride infusion (Magnan et al. 2001).

Tau decreased forskolin-induced insulin secretion only in the male ob group (Fig. 5b). Previously, our research group showed that Tau enhances PKA-induced Ca^{2+} influx and insulin secretion in islets isolated from control mice (Ribeiro et al. 2010). This amino acid regulates the activity and expression of the PKA pathway in different cell types (Chen et al. 2009; Pina-Zentella et al. 2012; Liu et al. 2013). Further investigation is needed to understand the modulatory action of Tau in the PKA pathway in islets from obese rodents, but the lower forskolin-induced increment insulin secretion in the obT group may contribute to decrease islet hypersecretion, since cAMP/PKA are also involved in glucose-induced insulin secretion (Dyachok et al. 2008).

A normalization of the inhibitory action of the SNS on insulin secretion was demonstrated in female obT islets (Fig. 5d). In contrast, several reports have shown that Tau may decrease norepinephrine release from SNS terminals, decreasing heart rate, blood pressure and improving baro-reflex sensitivity in different models of hypertension (Singewald et al. 1997; Hano et al. 2009). Tau decreased circulating epinephrine and norepinephrine levels, as well as adrenal epinephrine content in deoxycorticosterone acetate-salt rats (Fujita and Sato 1988; Sato et al. 1991). In all these studies, Tau did not alter SNS or sympathoadrenal function in control rodents (Fujita and Sato 1988; Sato et al. 1991; Singewald et al. 1997; Hano et al. 2009). Furthermore, poor data concerning the effects of Tau in ANS regulation in metabolism has been reported; a study in healthy subjects submitted to a high-cholesterol diet for 3 weeks showed that Tau supplementation decreased low-density lipoprotein levels and norepinephrine urinary excretion (Mizushima et al. 1996). Our data suggest that, in the endocrine pancreas, Tau preserves normal SNS actions, which prevents islet hyperfunction in obesity.

5 Conclusion

In summary, islets from ob mice presented a hypersecretory function under basal and stimulatory glucose concentrations, as well as following the activation of the potentiating M3/PKC and PKA pathways. Furthermore, islets from female ob mice showed an impaired SNS inhibitory action. Tau supplementation prevented islet

hypersecretion in male and female ob mice. This effect may be associated with the modulation of the PKC and PKA pathways in the male ob group. In female ob mice, Tau normalized the action of the cholinergic/PKC pathways and the inhibitory action of SNS. It is probable that Tau prevents the imbalance in the PNS and SNS action in the endocrine pancreas in the obesity, an effect that contributes to improve body nutrient homeostasis.

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Effects of a High Fat Diet and Taurine Supplementation on Metabolic Parameters and Skeletal Muscle Mitochondrial Function in Rats

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Abbreviations

HF	High fat
NEFA	Non-esterified fatty acids
Tau	taurine
TG	Triglycerides

1 Introduction

As obesity and a range of co-morbidities, such as insulin resistance, type 2 diabetes and cardiovascular disease, have become major public health challenges worldwide, research into prevention and alleviation of obesity has intensified. Insulin resistance may be induced in liver and skeletal muscle in various experimental animal models by high fat or high sucrose diets (Buettner et al. 2007; Kim et al. 1999) and many studies indicate that the mitochondrial function in these organs are changed when insulin resistance is present (Asmann et al. 2006; Kelley et al. 2002; Mogensen et al. 2007; Petersen et al. 2004). Most studies on mitochondria in the context of type 2 diabetes have been focused on the oxidative capacity, which was generally shown to be decreased (Schrauwen and Hesselink 2004) although others have also shown an increase (Iossa et al. 2002) or no change (Hoeks et al. 2011).

Taurine supplementation has been shown to prevent diet-induced weight gain and adiposity in rodents (Camargo et al. 2013; Nardelli et al. 2011) as well as

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improve blood lipid profiles in both rodents (Fukuda et al. 2011; Murakami et al. 1999; Nardelli et al. 2011; Yokogoshi and Oda 2002) and human subjects (Zhang et al. 2004) and it has been suggested that obesity induced taurine deficiency may create a vicious cycle promoting obesity even further (Tsuboyama-Kasaoka et al. 2006). Furthermore, taurine supplementation improves hyperglycemia and insulin resistance in OLETF rats (Harada et al. 2004; Kim et al. 2012; Nakaya et al. 2000) and reduces diabetic complications, including retinopathy, nephropathy, neuropathy, atherosclerosis, and cardiomyopathy (Ito et al. 2012). Interestingly, taurine seems to have a unique mitochondrial protective effect thought to be mediated by taurine mediated prevention of intracellular, and especially mitochondrial, calcium overload (El Idrissi and Trenkner 1999).

It is currently unknown whether or not taurine can prevent high fat diet induced changes in skeletal muscle mitochondrial function as this may be one possible mechanism by which taurine improves the metabolic state in insulin resistance and type 2 diabetes. Thus, the present study aims to examine the effect of taurine supplementation on glucose and lipid parameters as well as skeletal muscle mitochondrial function in a high fat diet rat model.

2 Methods

2.1 Chemicals and Solutions

Unless otherwise stated, reagents were purchased from Sigma-Aldrich, and were of analytical grade or better.

2.2 Animals

All experimental procedures described were approved by The Danish Animal Experiments Inspectorate (permit 2013-15-2934-00904) and by the local animal facility at the University of Copenhagen, Denmark. Male Wistar Hannover GALAS (HanTac:WH) rats (Taconic, Ejby, Denmark) were randomly assigned to three different diet groups for 12 weeks after weaning: Con) A control diet for the high fat diet containing starch and sucrose instead of fat (D12450B; Open Source Diets, New Brunswick, USA), HF) A high fat diet containing 60 % energy from fat (D12492; Open Source Diets) and HF+Tau) The high fat diet supplemented with 2 % taurine (w/v) in the drinking water. The taurine used was a chemically synthesized variant and was dissolved directly in the water used in the animal facility. The rats were fed ad libitum, except for the HF+Tau group which was pair fed with the

HF group, housed two rats per cage, and kept at a 12-h light/dark cycle. Animals and food intake was measured bi-weekly.

Rats were sedated with a mixture of Hypnorm (active ingredients fentanyl and fluanisone at a concentration of 0.079 mg/ml and 2.5 mg/ml, respectively) and Dormicum (active ingredient midazolam at a concentration of 1.25 mg/ml) in water given as 0.3 ml pr. 100 g of body weight. Following dissection, part of the quadriceps and liver were quick frozen in liquid nitrogen and stored at −80 °C for later analysis with the rest of the quadriceps used for isolation of mitochondria.

2.3 Plasma Parameters and Oral Glucose Tolerance Test

Triglycerides were measured in 50 mg tissue or in 10 μl plasma, hydrolyzed in 0.5 M KOH/85 % ethanol at 60 °C for 30 min. After cooling, $MgSO_4$ was added to 0.1 M and samples were vortexed and centrifuged at 16,000 g for 20 min at 4 °C followed by measurement of glycerol spectrophotometrically at 340 nm as described (Wieland 1984). Plasma Non-Esterified Fatty Acids (NEFA) were measured at 546 nm using NEFA-HR (2) Kit according to instructions from the manufacturer (WAKO, Richmond, VA, USA) at 37 °C.

Taurine content was measured in 10 μl plasma or 50 mg tissue, by homogenization in 10 % (w/v) TCA, followed by neutralization with 1 M KOH in 100 mM Imidazole buffer. Taurine content was measured spectrophotometrically as described (Matsuda and Asano 2012).

After 11 weeks on the respective diets—following an overnight fast (16–18 h), rats were weighed, a tail vein punctured using a lancet (BD Microtainer, Contact-Activated Lancet, 2.0 mm × 1.5 mm, BD biosciences, Erembodegem, Belgium), baseline (fasting) plasma glucose was measured using plasma calibrated strips (Accu-Chek Compact Plus, Roche, Basel, Schweiz) and a blood sample (~200 μl) collected for EDTA-plasma (Microvette CB 300, Sarstedt, Nümbrecht, Germany). Rats were gavaged a 2 g/kg body weight dose of glucose from a 22.5 % (w/v) glucose solution using polyethylene tubing (Fuchigami Kikai, Kyoto, Japan). At 30, 60, 90 and 120 min after gavage, plasma glucose was measured from fresh tail punctures as above. All measurements were performed in duplicate using two different glucometers.

2.4 Mitochondria

Mitochondria were prepared as described in (Fritzen et al. 2007; Jørgensen et al. 2012) from freshly dissected quadriceps skeletal muscle resulting in a suspension of mitochondria containing 3–5 mg protein per ml.

Muscle mitochondria preparations were tested for outer membrane integrity by observing the changes of the addition of cytochrome c during state 3 respiration. Only preparations with negligible changes were included in the study.

Mitochondrial function was tested as described in (Jørgensen et al. 2012). In brief: Oroboros Oxygraph-2 K instruments (Oroboros Inc., Austria) were used at 25 °C. 10 μl mitochondrial suspension was added to 2 ml of medium in each chamber. Stirrer speed was 600 rpm. Approximately 5 min after the mitochondria were introduced into the chambers, malate plus pyruvate or malate plus palmitoyl carnitine was added to final concentrations of 2 mM, 0.5 mM and 2 mM and 10 μM, respectively. Once a steady state 4 respiration was observed, ADP (3 mM) was added to obtain a state 3 respiration. Between experiments the chambers were washed twice with ethanol and water.

2.5 Statistical Analysis

Significant differences were evaluated using student's t-tests using SAS 9.2 (The SAS Institute, Cary, NC, USA). The level of significance was set at $P<0.05$.

3 Results

3.1 Physiological and Plasma Parameters

The high fat diet resulted in a significant increase in body weight after 4 weeks of feeding that persisted throughout the experiment until 12 weeks in both HF and HF+tau groups compared to Con (Fig. 1 and Table 1). The HF and HF+tau (pair fed with the HF group) groups had a significantly higher calorie intake than Con. No effect of taurine on body weight increase or food intake was observed (Table 1).

High fat diet caused a marked glucose intolerance in both HF and HF+tau groups compared with Con, with no effect of taurine (Fig. 2). There was no difference between groups with regard to fasting plasma glucose, free fatty acids or triglycerides.

3.2 Tissue Parameters and Mitochondrial Function

A marked increase in hepatic triglyceride content, but not in skeletal muscle, was observed in the HF group. Interestingly, taurine reversed the high fat diet induced increase in hepatic triglyceride content, but had no effect on skeletal muscle triglyceride content (Table 1).

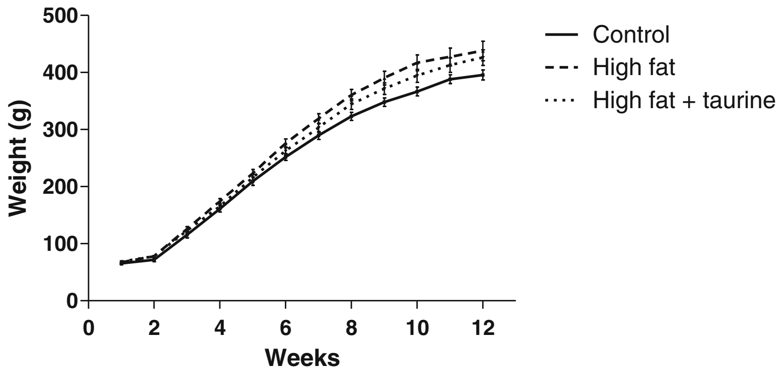

Fig. 1 Taurine does not prevent high fat diet induced weight gain. Wistar rats (N = 6) per group were given a control diet, a high fat diet or a pair fed high fat diet supplemented with 2 % taurine in the drinking water for 12 weeks directly after weaning.

Table 1 Physiological, plasma and tissue parameters and skeletal muscle mitochondrial respiration at 12 weeks

	Group		
Parameter	Control	High fat	High fat + taurine
Body weight (g)	396 ± 9	438 ± 17[a]	426 ± 14[a]
Food intake (kcal)	4785 ± 24	5293 ± 131[a]	5112 ± 79[a]
Plasma glucose (mM)	5.1 ± 0.3	5.7 ± 0.2	5.6 ± 0.2
Plasma NEFA (mM)	0.79 ± 0.17	0.86 ± 0.12	1.12 ± 0.06
Plasma TG (mM)	0.44 ± 0.08	0.33 ± 0.09	0.46 ± 0.09
Liver TG (mmol/g)	25.1 ± 2.4	81.6 ± 8.0[a]	33.3 ± 5.9[b]
Muscle TG (mmol/g)	14.2 ± 3.8	7.2 ± 2.5	8.6 ± 1.6
Plasma taurine (μM)	79.6 ± 9.1	104.6 ± 20.9	159.8 ± 31.9[a]
Liver taurine (mmol/g)	9.3 ± 1.5	14.7 ± 0.8[a]	33.8 ± 1.7[a,b]
Muscle taurine (mmol/g)	26.3 ± 2.4	23.4 ± 1.1	30.5 ± 1.0[b]
Muscle state 3 respiration, pyruvate (nmol O_2/min/mg protein)	77.1 ± 10.2	128.5 ± 16.3[a]	151.3 ± 9.3[a]
Muscle state 3 respiration, PC (nmol O_2/min/mg protein)	63.2 ± 9.7	85.3 ± 11.6	93.8 ± 6.2

Wistar rats (N = 6) per group were given a control diet, a high fat diet or a pair fed high fat diet supplemented with 2 % taurine in the drinking water for 12 weeks directly after weaning. NEFA; Non-esterified fatty acids, TG; Triglycerides

[a]Significantly different from control

[b]Significantly different from high fat

The high fat diet increased the taurine concentration in liver, but not in plasma or skeletal muscle in the HF group compared with Con. Taurine supplementation in the HF + tau group further increased the liver taurine concentration when compared to HF as well as increased plasma taurine levels when compared to Con (Table 1).

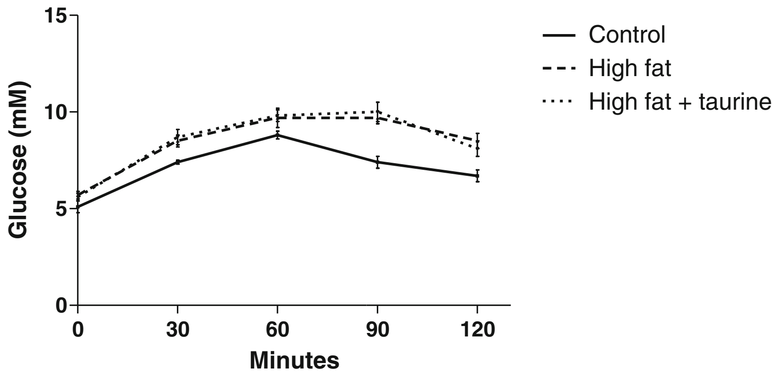

Fig. 2 Taurine does not prevent high fat diet induced glucose intolerance. Wistar rats (N=6) per group were given a control diet, a high fat diet or a pair fed high fat diet supplemented with 2 % taurine in the drinking water for 12 weeks directly after weaning. An oral glucose tolerance test was performed as described in methods

A significant increase in skeletal muscle mitochondrial state 3 respiration, with pyruvate as substrate, was observed in both the HF and HF+tau groups compared to Con, with no effect of taurine supplementation. No difference between groups was observed when examining skeletal muscle mitochondrial state 3 respiration using palmitoyl-carnitine as substrate (Table 1).

4 Discussion

The present study set out to examine the effect of taurine supplementation in rats receiving a high fat diet upon glucose and lipid parameters as well as skeletal muscle mitochondrial function

4.1 Taurine, High Fat Diet and Mitochondrial Function

Taurine has in studies from several different laboratories been shown to counteract the effects of a high fat diet on both glucose and lipid metabolism in rodent, with no observable negative side effects of taurine supplementation (Fukuda et al. 2011; Murakami et al. 1999; Nardelli et al. 2011; Yokogoshi and Oda 2002). In the current study, we failed to notice an improvement in glucose tolerance or body weight increase. However, taurine did normalize the hepatic triglyceride content indicating an interaction between taurine and liver lipid metabolism or lipid storage. Interestingly, this is the exact same observation we previously obtained in another model of diet induced insulin resistance, the fructose fed rat (Larsen et al. 2013).

The differences between these and previous studies showing effects on both glucose tolerance, weight gain and insulin resistance, could be due to differences in animals, or, more likely, differences in the dietary composition and taurine amount supplemented. In support of our findings it is noticeable that we see a clear increase in taurine availability in both plasma, liver, and skeletal muscle indicating that our taurine supplementation scheme did indeed work as planned. Furthermore, the animals given a high fat diet showed a clear glucose intolerance and weight gain, something which could not be prevented by the increased taurine levels.

In the present study, insulin concentrations were not measured. As taurine has been shown to potentiate insulin release (Carneiro et al. 2009), it is possible that the insulin levels during fasting and oral glucose tolerance test were changed in the HF+tau group and potential beneficial or detrimental effects of taurine could thus be hidden from view.

High fat diet induced an increase in skeletal muscle state 3 respiration using pyruvate as substrate, in agreement with some previous findings (Iossa et al. 2002). Interestingly, others have found that a high fat diet increase respiration when using lipids as substrate (Turner et al. 2007), something we could not corroborate in the present study although this may be due to differences in dietary composition. However, we did not observe an effect of taurine upon mitochondrial respiration, despite previous observations that taurine is important for mitochondrial respiration activity of the electron transport chain (Jong et al. 2010; Suzuki et al. 2002; Warskulat et al. 2006). On the other hand, an effect of taurine upon mitochondrial function and/or protection of mitochondria has mainly been shown in the context of taurine depletion. However, in the current study, we did not observe any taurine depletion in skeletal muscle and it may be speculated that as long as a threshold of taurine sufficiency is reached, further taurine supplementation will not have an effect on mitochondrial function.

5 Conclusion

In summary, the current study indicates that taurine supplementation may be able to counteract a high fat diet induced fatty liver as taurine caused a normalization of hepatic triglyceride content. However, we observed no effect of taurine upon skeletal muscle mitochondrial function despite a high fat diet induced increase in state 3 respiration. The lack of a taurine effect on mitochondrial respiration may indicate that once a threshold of taurine sufficiency is reached, more taurine does not improve or protect mitochondrial function.

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Taurine and Regulation of Mitochondrial Metabolism

Svend Høime Hansen, Henrik Birkedal, Flemming Wibrand, and Niels Grunnet

Abbreviations

ACAD	Acyl-CoA-dehydrogenase
CS	Citrate synthase
PDH	Pyruvate dehydrogenase

1 Introduction

During the last decade an increasing focus has been put on the role of taurine in the mitochondria of animals. As taurine is found in higher concentrations in oxidative tissue (20–50 mM) than in glycolytic tissue (5–10 mM), localisation in a sub-cellular compartment like the mitochondria is supported by a simple two-compartment model (Hansen et al. 2006). Experimental evidence for mitochondrial localisation has been obtained, e.g. NMR studies of isolated mitochondria demonstrated taurine localisation (Bollard et al. 2003) and analysis of isolated mitochondria found concentrations of about 70 mM (Jong et al. 2010). Finally, taurine-modifications of

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mitochondrial tRNA have been reported (Suzuki et al. 2002). Consequently, taurine must be present in the mitochondrial matrix.

The unusual high mitochondrial taurine concentration could be understood when considering taurine as a mitochondrial matrix pH buffer (Hansen et al. 2006, 2010; Hansen and Grunnet 2013). Mitochondrial oxidative function is today understood and explained on the basis of the chemiosmotic theory originally formulated by Peter Mitchell in the 1960s (Mitchell 1966, 1968) and today presented in condensed form in all biochemical text books and in more detailed form in presentations focused on the bioenergetics (e.g. Nicholls and Ferguson 2013). However, it seems that most of these modern versions of the chemiosmotic theory focus on the potential and proton-motive force energy as the driving force for proton pumping by the membrane-localised electron transport chain enzymes with creation of a mitochondrial pH gradient across the inner-membrane. Finally, the protons are employed as substrates for the ATP production by ATP synthase, which is also a membrane localised enzyme. Most, if not all, presentations overlook the importance of the mitochondrial pH buffering capacities as stated by Mitchell in the original theory (Mitchell 1966, 1968; Mitchell and Moyle 1967). Using a rechargeable electric battery as an analogue to the mitochondria, the electric potential represent the voltage of the battery, whereas the pH buffering capacities gives rise to the electrical working capacity of the battery. Even the mitochondrial redox systems of the mitochondria like glutathione and thioredoxin could be considered as very tightly coupled to the pH regulation. The ionisation constants (p*K* values) of taurine and the thiol groups in glutathione seem to be almost identical (Hansen and Grunnet 2013). Consequently, regulation and maintenance of pH buffering of the mitochondrial inner-compartments are vital for mitochondrial oxidative function.

In parallel, taurine has been found to have a role in the mitochondrial protein synthesis due to its modification of tRNA. Actually a direct role in expression of a protein in the electron transport chain has been observed (Jong et al. 2012). Thus, by taurine depletion, the activity of Complex I is downregulated resulting in a reduced capacity for mitochondrial proton pumping. However, the two lines of models compliment each other perfectly as hand in glove. Logically, the capacity for proton pumping needs to be closely correlated with a sufficient mitochondrial pH buffering capacity.

2 Methods

2.1 Determination of Enzyme pH Profiles

Citrate synthase. Activity was assessed at 37 °C in a final volume of 0.2 ml using a SPECTRAmax PLUS384 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) essentially as described (Srere 1969) in a medium containing 0.5 mM oxaloacetate, 0.2 mM acetyl-CoA, 0.1 mM 5,5′-dithiobis-2-nitrobenzoic

acid, 14 U/l citrate synthase (from pig heart, Roche, Basel, Switzerland), and for pH buffering 43.5 mM of either Tris or taurine. The buffer solutions were thermostated at 37 °C and then adjusted to the required pH with HCl or NaOH. Activity was determined from the linear increase in absorbance at 412 nm. Each point in the figure is the mean ± S.E. of five to eight measurements.

Pyruvate dehydrogenase. Enzyme activity was assessed at 37 °C in a final volume of 0.2 ml using a PowerWave XS microplate spectrophotometer (BioTek Instruments, Winooski, VT, USA). The assay was performed essentially as described in Brown and Perham (1976). The medium contained 4.8 mM sodium pyruvate, 5.7 mM $MgCl_2$, 0.3 mM $CaCl_2$, 8.6 mM Thiamine pyrophosphate (TPP), 0.3 mM CoA, 9.5 mM NAD^+, 7.4 mM cysteine, 0.01 U/ml pyruvate dehydrogenase purified from pig heart (Sigma), and for pH buffering 64 mM either taurine or Tris. The buffer solutions were thermostated at 37 °C and then adjusted to the required pH with HCl or NaOH. Activity was determined from the linear increase in absorbance at 340 nm. Each point in the figure is the mean ± S.E. of five to eight measurements.

3 Results and Discussion

3.1 Taurine as Mitochondrial pH Buffer

Stabilising the pH gradient by the presence of a buffer compound like taurine in the mitochondrial matrix actually transforms the matrix into a biochemical reaction chamber with a slightly alkaline pH 8.0–8.5 (Hansen et al. 2010). Several different techniques have been applied for demonstrating the existence of the pH gradient across the mitochondrial inner-membrane (Nicholls and Ferguson 2013). Application of techniques combining confocal microscopy with fluorescent protein techniques on mammalian cells has reported a mildly alkaline pH value for the mitochondrial matrix of 7.9–8.4 and for the cytosol 7.2–7.4 (Llopis et al. 1998; Cano Abad et al. 2004), or pH = 7.6 for the cytosol, 6.9 for the mitochondrial intermembrane space and 7.8 for the matrix (Porcelli et al. 2005). However, the obtained pH values might be a consequence of the chosen experimental conditions, e.g. the choice of pH buffer as seen in one of the studies (Porcelli et al. 2005).

It is well established that several important biochemical processes occur only inside the mitochondrial matrix, e.g., several of the reactions of the tricarboxylic acid cycle, β-oxidation of fatty acids and two of the reactions in the urea cycle. In experimental studies of enzymatic reactions, a number of different buffers are routinely used for controlling pH drifts resulting from the enzyme activity. However, to our knowledge studies on mitochondrial enzymes employing a buffer that mimics closely physiological condition are lacking. Instead, such studies use non-physiological biological buffers like Tris, triethanolamine, MOPS or HEPES. This fact can affect the enzyme activities, pH activity profiles and pH optima. An overview of these compounds can be found elsewhere (Hansen et al. 2010).

3.2 Metabolic Substrate Selection and pH Activity Profiles of Mitochondrial Enzymes

A general property of metabolic regulation is metabolic flexibility, i.e. the ability of the cell to switch between oxidation of carbohydrates and fatty acids, depending on the substrate supply and the energy requirement of the cell. This selection of substrates depends on the interplay of a number of regulatory mechanisms of the involved enzymes, but also the matrix pH may be important for the proper function of regulatory mechanisms involved in metabolic flexibility.

The pH activity profile of citrate synthase has previously been obtained in a classical study at 22 °C in Tris/acetate and imidazole/acetate buffers (Kosicki and Srere 1961). The pH profile of PDH has previously been reported at 25 °C in Tris/HEPES-buffer (Pawelczyk et al. 1992). We decided to obtain similar profiles, but changing the temperature to 37 °C and use two different buffer systems (Tris and taurine). The enzyme activity of citrate synthase (CS) from the tricarboxylic cycle and pyruvate dehydrogenase (PDH) regulating glucose oxidation were studied at 37 °C in two buffer systems (taurine and Tris) as shown in Fig. 1.

The data in Fig. 1 for CS and PDH demonstrate that the pH optima at 37 °C is in the pH range 8.0–8.5 for both enzymes and in both buffer systems. The pH optima observed coincide with the expected pH in the mitochondrial matrix and are found in the pH range with maximum buffering capacity of taurine as buffer due to its p*K*a = 8.6 at 37 °C (Hansen et al. 2010). The pH/activity profiles are narrower in taurine buffer compared with in Tris buffer. These minor observed differences might be attributed to enzyme interactions with the specific buffer compound.

We have previously reported a similar enzyme activity study for isocitrate dehydrogenase in Tris and taurine buffer at 37 °C (Hansen et al. 2006). The pH activity profiles were virtually identical for the two buffer systems with a pH optimum at 7.7–7.9, i.e. slightly lower than for citrate synthase. These results agree well with another report (Kim et al. 1999) on the maximal activity of mouse and human recombinant isocitrate dehydrogenase at pH 7.6–8.0 in MOPS/triethanolamine buffer. The observed pH optimum was also reported to depend on the tissue-specific isoform of the enzyme.

The two mitochondrial matrix enzymes from the urea cycle, ornithine transcarbamoylase (OTC) and carbamoylphosphate synthetase (CPS) have been isolated from human liver and some of their properties assessed in triethanolamine buffer at 37 °C. For OTC the pH optimum is 7.7, with only a modest 15 % decrease at pH 8.5 (Kalousek et al. 1978). For CPS maximum activity is at pH 7.8, and the enzyme activity decreases by 50 % at pH 7.0 and pH 8.5 (Pierson and Brien 1980).

The most important mitochondrial enzymes of the fatty acid degradation in the mitochondrial matrix are the acyl-CoA dehydrogenases (ACADs) as they control the β-oxidation of the fatty acids. Examples of pH activity profiles for these enzymes can be found elsewhere, either obtained in Tris or taurine buffers (Ghisla and Thorpe 2004; Hansen et al. 2010). These enzymes exhibit a rather low activity when pH < 7, but the activity increases steeply in the pH range from 7 to 9. Actually, the activity

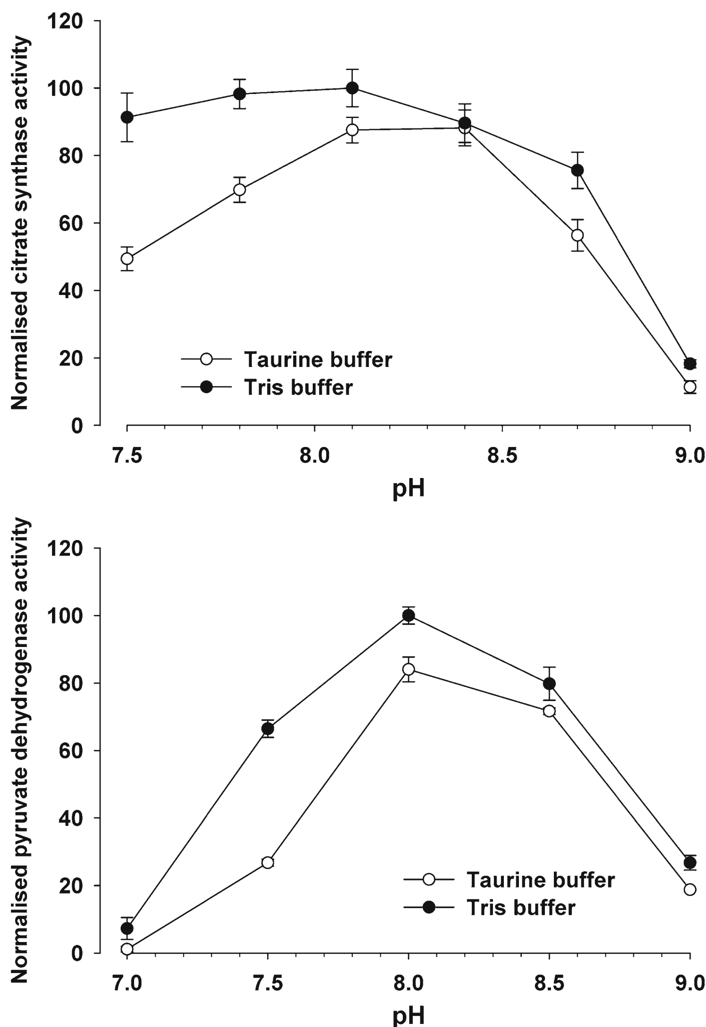

Fig. 1 Comparison of pH dependences of citrate synthase activity and pyruvate dehydrogenase at 37 °C using Tris (*filled circles*) or taurine (*open circles*) buffers. Each point is the mean ± S.E. of five to eight measurements

increases 10–20-fold from pH = 7 to pH = 9 for the ACAD enzymes studied, so β-oxidation strongly depend on having a slightly alkaline environment in the mitochondrial matrix.

Furthermore, the pH optima for the ACADs are found at a higher pH than the optimum for PDH. This means that in the pH range 8.0–8.5, even minor changes in the matrix pH might contribute in determining the preferred metabolic substrate for production of acetyl-CoA for further degradation in the tricarboxylic acid cycle.

Table 1 pH dependence in the mitochondrial matrix of favoured enzyme systems and associated substrate oxidation

Mitochondrial matrix pH	Favoured enzyme systems	Substrate selection
Low (pH 7.0–8.0)	Urea cycle: ornithine transcarbamoylase (OTC), carbamoylphosphate synthetase (CPS)	Amino acids
Medium (pH 7.5–8.5)	Tricarboxylic acid cycle enzymes, pyruvate dehydrogenase	Glucose/carbohydrates
High (pH 8–9)	Acyl-CoA dehydrogenases	Fatty acids

These observations clearly indicate that the regulation of glucose and fatty acid metabolism requires adequate pH buffering of the mitochondrial matrix in the pH range 8.0–8.5, possibly provided by taurine with its characteristics as ideal buffer compound in this pH range, but taurine also prevents excessive alkalinisation of the matrix. If the matrix becomes too alkaline, i.e. about pH 9, pyruvate dehydrogenase and citrate synthase will both become impaired with subsequent production of acetyl-CoA due to the continued β-oxidation. This in turn may cause a relative inhibition of β-oxidation and accumulation of fatty acids and triglycerides with adverse metabolic effects.

To summarize, available information on mitochondrial matrix enzymes indicates activity optima at pH values in the range from 7 to about 9. The urea cycle enzymes have optima in the lower pH range, then PDH and tricarboxylic acid enzymes in the central range and finally the β-oxidation in the high range of this pH interval. A brief summary on the relationship between mitochondrial matrix pH and the favoured metabolic enzyme systems and thus substrate selection is given in Table 1.

3.3 *Hypothesis: Taurine as pH Dependent Regulator of Pyruvate Dehydrogenase Kinase*

It is well-known that the regulation of PDH activity is very complex, as the enzyme is presented as an enzyme complex with several associated kinases and phosphatases as activity regulators (Sugden and Holness 1994, 2003). In general, PDH will be inhibited when phosphorylated by the kinases, so inhibiting the kinases will maintain the pyruvate oxidation by PDH and lead to continued ‘normal’ glucose metabolism.

Interestingly, several studies from the 1990s by Barry Lombardini demonstrated a possible involvement of taurine in the regulation of the PDH kinases (e.g. Lombardini 1994, 1997). The studies identify a specific 44 kDa kinase, which presumably is one of the pyruvate dehydrogenase kinases, which is specifically inhibited by taurine in millimolar concentrations, i.e. in the possible physiological concentration range.

However, the studies do not consider the slightly alkaline pH of the mitochondrial matrix, so the inhibition only reflects the zwitterionic form of taurine ($^{+}H_3NCH_2CH_2SO_3^{-}$),

not the alkaline deprotonated anionic form taurate (2-aminoethane-1-sulfonate, $H_2NCH_2CH_2SO_3^-$). It may therefore be hypothesised that taurate does not have any inhibitory function on the PDH kinase. With increasingly alkaline pH, taurate would no longer inhibit the PDH kinase and due to phosphorylation of PDH, the enzyme and thus glucose metabolism would decrease. This would be in perfect agreement with the active lipid β-oxidation at alkaline pH. Indirect support for this suggestion and the different roles of the taurine buffer pair compounds can be found in the classic inhibition studies with taurine analogues, as the hydroxyl analogue of taurine (2-hydroxyethane-1-sulfonic acid: $HOCH_2CH_2SO_3H$, although probably in the anionic form as 2-hydroxyethane-1-sulfonate: $HOCH_2CH_2SO_3^-$) was reported not to exert any inhibitory function of the PDH kinase (Lombardini 1994).

3.4 Metabolic Flexibility and Diabetes

During the last decades the research of metabolic substrate selection and regulation has been intense. Special focus has been put on the understanding of the metabolic switch, often called the Randle cycle, between the different types of substrates, as the lack of such metabolic flexibility is a basic characteristic of the metabolic syndrome and type 2 diabetes (Randle 1998; Kelley et al. 1999; Bergman et al. 2007; Galgani et al. 2008; Hue and Taegtmeyer 2009). The involvement of two independent enzyme systems in the glucose and lipid metabolism introduces the obvious possibility of having some kind of external mediator to assist in the metabolic regulation. Surprisingly little focus seems to have been given to the relationship between the pH in the mitochondrial matrix and its consequences on the activity of the underlying metabolic enzyme systems. But unfortunately performing functional metabolic studies with controlled matrix pH will be almost impossible.

Here we have presented a very simple model pointing at a possible relationship between the matrix pH and activity regulation of important metabolic enzyme systems. Taurine and taurate has been suggested to act as mitochondrial matrix buffer-system, but the buffer pair could possibly also be key regulators in the PDH kinase systems with the matrix pH to determine the relative proportion of the buffer pair and thus the subsequent inhibitory action towards the PDH kinase.

Major alterations in the taurine concentration, e.g. as a consequence of depletion, could cause changes in the metabolic flexibility between glucose and fatty acids. Actually depleted taurine levels could cause inadequate inhibition of the PDH kinase and thus deactivation of PDH, but at the same time low taurine levels could lead to insufficient pH buffering capacity for having a stable β-oxidation. Consequently, glucose and lipid metabolism would simultaneously be down-regulated, resulting in dysfunctional metabolic flexibility, which is a typical characteristic in type 2 diabetes. So perhaps the role of taurine represents one of the unwritten chapters in the review of the Randle cycle (Hue and Taegtmeyer 2009)?

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Part IV
Taurine and the Central Nervous System/ Taurine: Endocrine System and Locomotor Activity

Open Questions Concerning Taurine with Emphasis on the Brain

Simo S. Oja and Pirjo Saransaari

1 Introduction

Taurine was discovered almost 200 years ago and it and its functions have been subjected to fairly intense research during the last decades. However, there are still several matters open or in need of more thorough investigation. We briefly summarize these in this article, concentrating mainly on taurine and the brain because of our half-a-century-long career in studies of taurine in this context.

2 Taurine Biosynthesis

Taurine is formed in the mammalian organisms through two main pathways, both originating from cysteine. One of them proceeds through cysteinesulphinate, which is transformed to hypotaurine by means of sulfinoalanine decarboxylase (EC 4.1.1.29). The other involves cysteamine, which is oxidized to hypotaurine by cysteamine dioxygenase (EC 1.13.11.19). In both metabolic pathways hypotaurine is the central intermediate. Most studies on taurine biosynthesis leave off at this stage and hypotaurine is taken in some obscure manner to be converted to taurine. This last step has not been satisfactorily explored. A putative hypotaurine dehydrogenase was first suggested to exist by Sumizu (1962). However, when we attempted to reproduce his findings, it appeared impossible. We obtained some results, however, which indicated that the conversion was enzymatic (Oja and Kontro 1981). It has also been suggested that oxidation of hypotaurine to taurine might occur via a nonenzymatic

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reaction (Fellman et al. 1987). This proposition has since been generally accepted without a convincing background of evidence. It is also true that no enzyme responsible to hypotaurine transformation to taurine has been purified to homogeneity and characterized. A few years ago Vitivsky and colleagues (2011) revisited this matter and obtained data showing that conversion of hypotaurine to taurine in primary astrocytes and neurons is enzyme-catalyzed and regulated, constituting possibly the rate-limiting step in taurine biosynthesis. The enzyme possibly involved still remains uncharacterized. Some important questions arise. Why has the enzyme involved in the conversion of hypotaurine to taurine remained so obscure, if this step is enzymatic? On the other hand, if taurine is indeed an important compound, why could its synthesis rely on a random non-enzymatic conversion?

3 Taurine Concentrations and Development

Taurine is present in all animal tissues and organs. In the brain it is generally one of the most abundant amino acids. Species differences are very considerable, however. For example, in the mouse and rat brain the taurine content is several tenfold greater than in the guinea-pig brain (Oja and Saransaari 2007). Maternal milks of different species likewise contain very different concentrations of taurine, For example human milk is enriched by taurine while cow milk is not. There obtains in different species a good positive correlation between taurine content in the brain and the taurine content in mother milk. How can different species manage with so different taurine concentrations?

In all species the taurine content in the developing brain is invariably greater than in the adult brain. The levels gradually diminish during postnatal development. This is in contrast to other amino acids, which tend to increase during the same period (Oja and Kontro 1983). The high taurine content in the developing brain may signify that it is especially important at that stage. This importance has been convincingly demonstrated in developing kittens (Sturman 1993). Cats cannot conjugate glycine, but only taurine, with bile acids as do rats and mice and are therefore susceptible to dietary taurine deficiency. In primates (Imaki et al. 1987) and humans (Ament et al. 1986) early taurine deficiency symptoms have also manifested themselves if their offspring are nourished with taurine-deficient baby formulas. The formulas prepared from taurine-poor cow's milk are therefore nowadays fortified with taurine. As stated above, taurine is essential during early development, but the exact mechanisms underlying this are not known.

4 Taurine and Osmoregulation

The role of taurine in osmoregulation in marine animals has been known for decades, but only in the 1980s was it suggested to have the same role in the mammalian brain (Walz and Allen 1987). Taurine has been shown in many studies to be released from

astrocytes and neurons in vitro by hypo-osmotic media (e.g., Pasantes-Morales and Schousboe 1997) and also in vivo (Solís et al. 1988). Taurine has been sometimes claimed to be a particularly suitable osmolyte in that it is metabolically inert and has no other specific biological functions. This last assumption is not true, however, as we discuss below. Nor is the enhanced release of taurine from neural cells invariably associated with cell swelling, being under certain conditions associated with intracellular shrinking in brain slices in vitro (Oja and Saransaari 1992) and in vivo (Phillis et al. 1999). Potassium stimulation has often been assumed to release taurine from brain cells solely due to excitation-induced cell swelling, but potassium-evoked release occurs also when swelling is prevented by impermeable anions (Oja and Saransaari 1992). It is thus open to what extent the release is induced under different conditions by depolarization of neural cells on one hand and by cell volume regulation on the other.

5 Taurine Effects on Neural Transmission

Taurine inhibits neuronal firing by introducing hyperpolarization via an increase in the membrane permeability to chloride ions (Saransaari and Oja 2008). These actions are in most cases blocked by antagonists of GABA or glycine, depending on the brain area. Taurine induces single-channel currents by opening Cl^- channels in cultured rat cerebellar granule cells in patch-clamp experiments (Linne et al. 1996). At low concentrations it inhibits GABA-induced Cl^- fluxes into synaptic membrane microsacs, but at higher concentrations enhances the fluxes, apparently due to activatory properties of its own (Oja et al. 1990).

It is difficult to determine whether the physiological functions of taurine are mediated by GABA and/or glycine receptors or whether specific taurine receptors are involved. We were the first to report taurine binding to mouse brain synaptic membranes (Kontro and Oja 1983), if the membranes were subjected to freezing-thawing cycles and detergent treatments to extract as much as possible of the endogenous taurine attached to the preparations tested (Kontro and Oja 1987a). Frosini and co-workers (2003) have shown that taurine binding to washed and detergent-treated synaptic membranes from the rabbit brain is not affected by GABAergic agents. The binding does not thus represent binding to GABA receptors. On the other hand, taurine displaces glycine from its strychnine-sensitive binding sites in the mouse brain stem (Kontro and Oja 1987b). Single point mutations in isolated glycine receptors have been shown to essentially alter their taurine sensitivity (Saransaari and Oja 2008). For example, the homomeric αZ1 receptor from the Zebra fish, which contains valine instead of isoleucine in position 111, exhibits exceptionally high sensitivity to taurine, almost comparable to glycine sensitivity (David-Watine et al. 1999). The cloned homomeric α4 subunit from chicks, containing valines in positions 111 and 212, is also potently activated by taurine (Harvey et al. 2000).

It remains open whether or not all taurine actions in neurotransmission are attributable to its interactions with GABA and glycine receptors. In particular, the cloning of different glycine receptors from various sources and their mutations,

which alter the responses to taurine, glycine and GABA, would suggest the existence of independent taurine receptors. It may be noted that the efficacy of taurine at glycine receptors appears to be greater in developing animals and in animal species other than mammals. Even in mammals some effects in certain brain areas could be mediated by receptors specific for taurine (Saransaari and Oja 2008). Could the molecular structure of such receptors be altered during development and do there exist significant differences in the structure in different animal species?

6 Summary

There are still a number of open questions concerning the role of taurine in the brain. We have briefly listed the most obvious of them in this article: the mechanism of conversion of hypotaurine to taurine, great species differences in taurine concentrations, the specific role of taurine in development, and its cell volume-regulatory and neuromodulatory or neurotransmitter actions.

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Taurine Supplementation Induces Hyperinsulinemia and Neuronal Hyperexcitability

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Abbreviations

Tau	Taurine
GAD	Glutamic acid decarboxylase
IR	Insulin receptor
IGF	Insulin-like growth factor
WT	Wild type controls

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1 Introduction

Taurine (2-aminoethanesulfonic acid) is a sulfur-containing amino acid. It is one of the most abundant free amino acids in many excitable tissues, including the brain, skeletal and cardiac muscles. Physiological actions of taurine are widespread and include bile acid conjugation, detoxification, membrane stabilization, osmoregulation, neurotransmission, and modulation of cellular calcium levels (Lambardini 1985; Solis et al. 1988; Foos and Wu 2002; Lourenco and Camilo 2002; Saransaari and Oja 2000; Schaffer et al. 2000). Furthermore, taurine plays an important role in modulating glutamate and GABA neurotransmission (Militante and Lombardini 1998; El Idrissi and Trenkner 1999, 2004). We have previously shown that taurine prevents excitotoxicity in vitro primarily through modulation of intracellular calcium homeostasis (El Idrissi and Trenkner 1999). In neurons, calcium plays a key role in mediating glutamate excitotoxicity. Taurine is added to milk formula and in solution for parenteral nutrition of premature babies to prevent retinal degeneration and cholestasis (Huxtable 1992; Lourenco and Camilo 2002). More recently, it has been shown that gestational taurine is able to prevent pancreatic alterations induced by gestational malnutrition, especially a low-protein diet (Dahri et al. 1991; Cherif et al. 1996; Merezak et al. 2001; Boujendar et al. 2002). In addition, taurine administration during gestation delays the mean onset time of diabetes in NOD mice (Arany et al. 2004); whereas taurine supplementation on dams fed with normal diet produces weak glucose intolerance, and increases islet sensitivity to cytokines in offspring (Merezak et al. 2001). Moreover, taurine plays a role in glucose metabolism in adults (Hansen 2001; Franconi et al. 2006).

As a potent anti-oxidant, taurine has a protective effect on the pancreas, presumably by preventing or scavenging free radicals. Previous reports propose the islets from taurine-treated mice had almost double the number of cells positive for proliferating cell nuclear antigen (PCNA). This increase proliferation is accompanied by a reduction in the incidence of apoptosis in islet cells, and also a significant increase in the number of islet cells immunopositive for IGF-II (Arany et al. 2004). Peak of islet cell apoptosis is maximal in the rat pancreas 14 days after birth and is temporally associated with a fall in the islet cell expression of IGF-II (Petrik et al. 1998). IGF-II functions as an islet survival factor *in vitro*. The induction of islet cell apoptosis *in vivo* may involve an increased expression of inducible nitric oxide synthase (iNOS) within β cells. Interestingly, taurine is a potent inhibitor of iNOS (Liu et al. 1998). Similarly, Scaglia et al. (1997) have shown decreased replication and an increased incidence of apoptosis in the β cells in the presence of IGF-II.

The functional significance of increased insulin production and secretion in response to plasma glucose challenges may be twofold. First, insulin may activate the IGF-II receptors expressed in the islets, which will further promote survival of β cells. Second, insulin, once in the brain, will activate IRs and cause hyperexcitability, which is observed in response to taurine supplementation.

Taurine acts as a partial agonist of $GABA_A$ receptors in synaptic membranes (Quinn and Harris 1995), and to activate Cl^- influx through $GABA_A$ receptors in

cerebellar granule cells *in vitro* (El Idrissi and Trenkner 2004). The interaction of taurine with $GABA_A$ receptors can also be shown *in vivo*. We have previously reported that subcutaneous injections of taurine (43 mg/kg) reduces seizure severity in mice injected with kainic acid (El Idrissi et al. 2003), suggesting that the anticonvulsive effects of taurine might be mediated by direct interaction with the $GABA_A$ receptors in vivo. Furthermore, the chronic interaction of taurine with $GABA_A$ receptors induces a variety of alterations to the GABAergic system that encompasses key proteins involved in synaptic transmission at the inhibitory synapse. These alterations include increased hippocampal and cortical GAD expression, decreased hippocampal expression of the beta 2/3 subunits of the $GABA_A$ receptor (El Idrissi and Trenkner 2004), and an increase in the number of somatostatin-positive neurons (Levinskaya et al. 2006). These biochemical changes to the inhibitory GABAergic system induced by taurine supplementation would affect the efficacy of the inhibitory system within the brain and render neuronal circuits more excitable. Coupled with these changes in the GABAergic system, here we report an increased expression and activation of the insulin receptors will further increase neuronal excitability in taurine-fed mice.

2 Methods

2.1 Animals

All mice used in this study were 2-month-old FVB/NJ males. For taurine-fed mice, taurine was dissolved in water at 0.05 %, and this solution was made available to the mice in place of drinking water for 4 weeks beginning at 4 weeks of age. All mice were housed in groups of three in a pathogen-free room maintained on a 12 h light/dark cycle and given food and water ad libitum. All procedures were approved by the Institutional Animal Care and Use Committee of the College of Staten Island/CUNY, and were in conformity with National Institutes of Health Guidelines. The number of mice used in these studies was sufficient to provide statistically reliable results.

2.2 Immunohistochemistry

Frozen sections were made as previously described (Levinskaya et al. 2006 and placed onto gelatin-subbed slides. Non-specific binding sites were blocked using 4 % bovine serum albumin (BSA), 10 % normal goat serum (NGS), and 0.05 % Triton X-100 in 0.01 M phosphate-buffered saline (PBS; pH 7.2). Following the blocking step, the slides were rinsed in an antibody dilution cocktail (ABD) consisting of 2 % BSA and 1 % NGS in 0.01 M PBS. Primary antibodies (Chemicon International)

employed were directed against insulin receptor (mouse host) diluted 1:500 in ABD. The primary antibody was incubated overnight at 4 °C and then unbound antibodies rinsed with ABD. Secondary antibodies were all raised in goat and directed against appropriate primary antibody type. The anti-mouse IgG was conjugated to Alexa Fluor 488 (Invitrogen/Molecular probes). Images were obtained by confocal microscopy (Leica SP2 AOBS). Nuclei were counterstained with SlowFade with DAPI (Invitrogen). To determine relative changes in protein expression, the gain and offset was identical for all comparisons. The intensity ratios of immunoreactivity were determined by importing the data from the Leica confocal software into Imaris X64 (Bitplane). For each Z stack, the threshold values for insulin receptor immunoreactivity were set for the untreated tissues. When the Z stacks for the taurine-treated tissues were imported, the Z stack were treated the same as the control. Coupling these manipulations with the consistent imaging parameters (same lens, gain and offset for each laser), the data changes are treatment-related. The mean pixel intensity values for each thresholded channel were obtained from the Imaris software and those data imported into InStat statistical software (GraphPad Software Inc.).

2.3 Intraperitoneal Glucose Tolerance Test

Mice from both groups were fasted overnight (12 h) and then injected intraperitoneally with 0.02 ml/g of body weight D-glucose (7.5 % stock solution in saline). Blood samples were taken by tail venesection at 0 min (just before glucose injection) and at 30-, 60-, and 120-min intervals after the glucose load. Glucose was measured with Ascensia Breeze portable glucose meter (Bayer, Germany). Mice were given only water during the test.

2.4 Statistic Analysis

Statistical significance was determined by Student's t-test. Each value was expressed as the mean ± SEM. Differences were considered statistically significant when the calculated P value was less than 0.05.

3 Results

3.1 Taurine-Fed Mice Exhibit Hyperinsulinemia and Glucose Tolerance

We have previously reported that taurine-supplemented mice have increase islets size and number. To determine the functional significance of these histological changes, we tested the tolerance of mice to glucose injection as an indicator of the

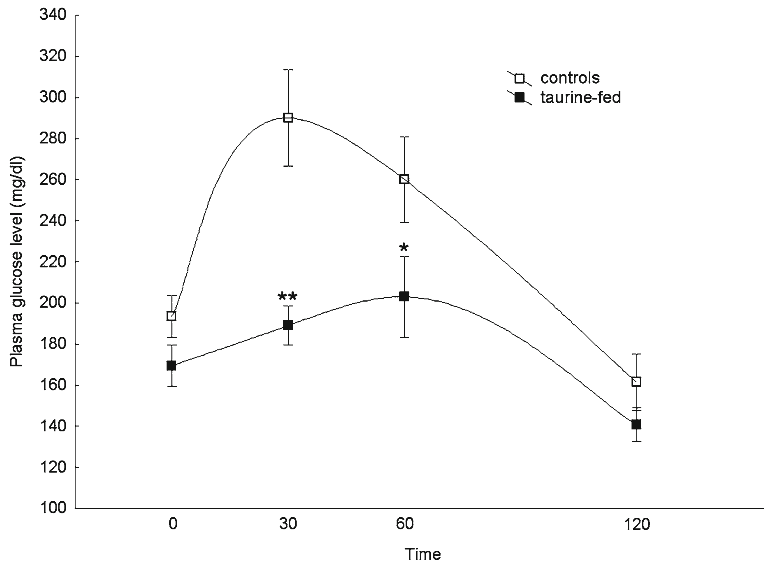

Fig. 1 Effect of taurine supplementation on glucose homeostasis. Intraperitoneal glucose tolerance test on overnight fasted control mice (n = 12) and taurine supplemented mice (n = 12). Values are expressed as means ± S.E.M obtained from three experiments. **$p < 0.01$, *$p < 0.05$ when compared with taurine group

pancreas efficiency to regulate plasma glucose homeostasis. As expected, control mice showed a drastic increase in plasma glucose concentration 30 min after challenge with a gradual decrease over through 120 min. By the end of the experiment, mice were slightly hypoglycemic relative to baseline (Fig. 1). In contrast, mice fed taurine showed a significant tolerance to glucose injection. Baseline plasma glucose levels indicated that these mice were slightly, but not significantly, hypoglycemic compared to controls. However, the response to glucose injection was drastically reduced ($p < 0.001$) at 30 min compared to controls. At 60 min following the challenge, the plasma glucose level in the taurine-fed mice rose slightly but was still significantly reduced over the controls at this time. Not until at 120 min post-challenge that the plasma glucose levels were similar in both groups.

3.2 Taurine-Fed Mice Have Increased Expression of Insulin Receptors in the Brain

To further investigate the functional significance of the histological changes occurring in the pancreas and the increased insulin production and secretion in response to glucose challenge, we examined the expression of the insulin receptor in the brain. Insulin is primarily a metabolic hormone functioning on muscle, fat and liver

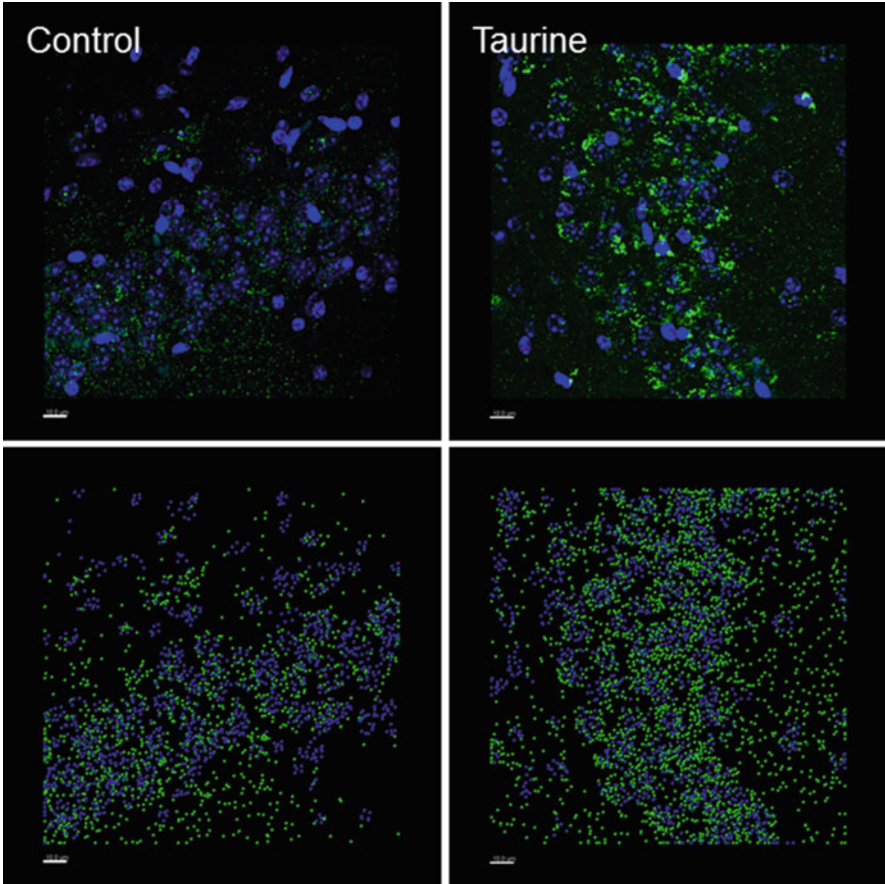

Fig. 2 Effect of taurine supplementation on insulin receptor expression in the hippocampus. *Upper panel* representative confocal images showing insulin receptor (*green*) immunoreactivity in CA3 region of the hippocampus from control and taurine-fed mouse, respectively. *Lower panel* depicts the Imaris reconstruction of the z-stacks. Hippocampi from taurine-fed mice show a significant increase in immunoreactivity for insulin receptor. Scale bar = μm

via activation of its cognate receptor, though it also functions on tissues that are not considered classically metabolic, such as the vasculature and the brain. Once insulin is secreted it crosses the blood-brain barrier by a transporter-mediated saturable mechanism. The IR is widely expressed in the brain but demonstrates denser expression in certain regions (Unger et al. 1991). A higher level of expression is found in the olfactory regions, amygdaloid complex, hippocampus, pyriform cortex and thalamus. This regional specify implicates insulin, through activation of its receptor, in various brain function that are mediated by these brain structures. In this study, we examined the levels of IR expression in the brain and found that taurine-fed mice have a significant increase in IR expression in all brain regions compared to controls (Fig. 2).

4 Discussion

We have previously shown that taurine-fed mice have a significant increase in the size of islets of Langerhans when compared to controls (El Idrissi et al. 2009). Concomitant with these histological changes, taurine-fed mice were tolerant to glucose challenges. When mice were injected with a glucose solution, taurine-fed mice handled plasma glucose load significantly better that controls (Fig. 1). Maintenance of glucose homeostasis by taurine-fed mice is attributable to the histological changes in the pancreas and the resulting increased insulin production and secretion by the pancreas of these mice when challenged with a glucose load.

Circulating insulin crosses the blood-brain barrier and bind to its cognate membrane-bound receptors on neurons. The IR is widely expressed in the brain but demonstrates denser expression in certain regions (Unger et al. 1991). A higher level of expression is found in the olfactory regions, amygdaloid complex, hippocampus, pyriform cortex and thalamus. Importantly, the IR is also highly expressed in the hypothalamus, specifically in the arcuate, supraoptic and dorsomedial nuclei (Havrankova et al. 1978). There is significant evidence that insulin mRNA is present in certain regions of the brain during development as well as in adult brain (Devaskar et al. 1993). Peripheral insulin enters the brain via a saturable mechanism involving the blood–brain-barrier, and it seems that the rate of entry varies according to the region.

There are numerous studies demonstrating that IR signaling plays a role in both excitatory and inhibitory neurotransmission, functions that are involved in higher brain functions. In addition, short- and long-term memory may affect IR expression levels in the rat hippocampus (Plum et al. 2005). Further support for a role of insulin in neuronal modulation is provided by studies showing that intranasal insulin delivery in mice leads to an increased expression of the potassium ion channel Kv1.3 in the olfactory bulb (Marks et al. 2009). Mice receiving intranasal insulin have improved cognition, as shown by short- and long-term object recognition. These findings suggest that insulin delivered to the CNS increases neuronal activity and improves memory by mechanisms involving changes in Kv1.3 levels. In addition to the role insulin plays in the adult brain, an important function for insulin in the CNS appears to be neuronal survival (Mielke et al. 2006). When rat hippocampal cells in culture are stressed by oxygen or glucose deprivation their survival can be rescued by insulin signaling through IR. Insulin also protects embryonic retinal cells during development from caspase and cathepsin-mediated apoptosis by inhibiting the expression of these pro-apoptotic proteins (Díaz et al. 1999).

The role of insulin receptor in fundamental biological processes (e.g., development, brain function, metabolism, etc.), along with more recent data linking brain insulin function to the etiology of a number of neurodegenerative diseases will, undoubtedly, translate into more clinically-oriented avenues of research in the near future.

5 Conclusion

In summary, the histological changes observed after taurine supplementation on the pancreas are consistent with the hypoglycemic effects of taurine and may have implication in diabetes. Additionally, taurine treatment resulted in a significant upregulation of insulin receptors in the brain. The regional distribution of insulin receptor in the brain coupled with the global upregulation may explain the wide neurobehavioral effects observed after taurine supplementation.

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Taurine Recovers Mice Emotional Learning and Memory Disruptions Associated with Fragile X Syndrome in Context Fear and Auditory Cued-Conditioning

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Abbreviations

a-Tau	Acute taurine
c-Tau	Chronic taurine
WT	Wild type control mice
KO	Fragile X knockout mice
CFC	Context fear conditioning test

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ACFCT Auditory cued fear conditioning test
FCX Frontal cortex
HP Hippocampus

1 Introduction

Taurine (2-aminoethanesulfonic acid) is a sulfur-containing amino acid abundant in mammalian excitable tissues such as: brain, skeletal and cardiac muscles. Physiological actions of taurine are widespread but here we will restrict our focus to neurotransmission, and neuromodulatory roles of glutamate and GABA neurotransmission (Militante and Lombardini 1998; El Idrissi and Trenkner 1999, 2004). It has been previously shown that taurine is neuroprotective against excitotoxicity via regulating intracellular calcium homeostasis (El Idrissi and Trenkner 1999). In neurons, calcium plays a key role in mediating glutamate excitotoxicity. In early development, taurine is critical for the development, growth and survival of neurons (Suge et al. 2007). Taurine occurs at high concentrations in the neonatal brain (Huxtable 1989, 1992; Sturman 1993), in contrast by adulthood the taurine levels reduce to levels below glutamate and further subside in senescence (El Idrissi et al. 2013). Thus, taurine may provide a compensatory role in mediating GABAergic related senescence (*For Review See*, El Idrissi et al. 2013) that may (1) recover age-related learning and memory problems, (2) increase the efficiency of the GABAergic system, (3) sustain levels of brain excitability comparable to LTP to facilitate learning when aged, and (4) provide neuroprotection during behaviorally relevant stressful conditions. Thus, as a function of age like many other neurochemical processes, GABAergic neurons undergo age-dependent neurodegeneration in which taurine supplementation may slow, reduce, and/or recovery the trajectory of memory related GABAergic cognitive decline (El Idrissi 2008). Thus, determining when to provide a pharmacotherapeutic intervention to ameliorate GABAergic senescence is of particular value. Information regarding taurine counteracting age-dependent memory is known, however far less is known of taurine influences on emotional memory and age-dependent effects on emotional based-behavioral signatures. We previously showed that acute (a-Tau) and chronic (c-Tau) administration in aged mice resulted in stress induced taurine differences associated with fear learning and emotional memory in WT FVB/NJ mice (Neuwirth et al. 2013). We postulated that a-Tau exposure produced enhanced fear and decreased inhibition to aversive stimuli in normal FVB/NJ mice (i.e. resulting in a mouse model of hyper-sensitivity /hyper-reactivity). Taurine levels in the brain have been reported to increase in response to stressors to offer neuroprotection against brain over-excitation (Riback et al. 1993; Wu et al. 1998; Suge et al. 2007). To expound upon our prior results we examined the effects of fear learning induced stressors in the Fragile X Syndrome mouse, an established model of hyper-excitability/hyper-arousal (El Idrissi et al. 2011). The Fragile X knock out mouse (KO), exemplifies the most inheritable form of intellectual disability characterized by a reduced efficiency in GABAergic signaling (El Idrissi et al. 2011) that further reduces with age as a promising face

and construct valid model. Here we assessed whether or not the $GABA_{AR}$ agonist taurine, dependent upon supplementation (i.e. acute and chronic), could stabilize and potentially recover the GABAergic emotional learning and memory deficits (i.e. age or genetic) consistent with our prior observations in normal FVB/NJ mice (Neuwirth et al. 2013) and the KO mouse model (El Idrissi et al. 2011). Moreover, in accordance with our studies in hippocampus where taurine regulated short term synaptic plasticity in Fragile X mice (El Idrissi et al. 2010), we examined the effects of [2,2 3^{H}]-Taurine uptake and release in frontal cortical and hippocampal synaptosomes following behavioral testing to elucidate neurochemical signaling underlying emotional experiences and how taurine supplementation differentially contributes to stress related neuroprotection from brain over-excitability in this model.

2 Methods

2.1 *Subjects and Treatment*

Experimentally naive FVB/NJ mice were used in accordance with The College of Staten Island (CUNY) IACUC approval procedures. The Fmr1 KO mutation was originally engineered in E14 embryonic stem cells derived from the 129/Ola strain (Bakker et al. 1994). FVB/NJ-129 hybrid mice carrying this mutation were obtained (a generous gift, B. Oostra and R. Willemsen) and repeatedly backcrossed to FVB/NJ at the CSI animal facility. KO and control littermates used in these experiments were the product of >20 generations of this process. Male mice were maintained under controlled temperature (30 ± 2 °C) and humidity (54 ± 3 %), on a 12-h light (7:00–17:00 h): 12-h dark (17:00–7:00 h) cycle. Food and water were available *ad libitum*. Taurine was either injected acutely (a-Tau: 43 mg/kg/s.c.) 15 min prior to testing or supplemented chronically in the drinking water (c-Tau: 0.05 % w/v) when the mice were 4 weeks old and continued for 4 months until testing between 6 and 7 months of age. Subject sample sizes Cont $N=12$ and KO $N=12$ were randomized as follows: Cont $N=4$, a-Tau $N=4$, c-Tau $N=4$, KO $N=4$, KO a-Tau $N=4$, and KO c-Tau $N=4$. Post testing only Cont, c-Tau, KO and KO c-Tau mice were immediately anesthetized, sacrificed and the frontal cortices' and hippocampi were extracted for synaptosomal physiological processing of taurine neurotransmission.

2.2 *Context Fear and Auditory Cued Conditioning*

The context fear and auditory cued conditioning paradigm was identical to the procedures used by Neuwirth et al. (2013) for mice to associate a learned tone with foot shock evidenced by increased freezing behavior vs. baseline. Briefly, mice were handled 1 h per day for 1 week prior to experimentation. Following handling mice were acclimated to the test holding and testing room for 1 h in red light (100 W/30 lx) and 5 min in green light (100 W/23 lx) respectively. Post acclimation mice were placed into the context fear-conditioning chamber (Med Associates, VT) and testing

was completed in green lighting. Testing was conducted in phases across 3 days. (a) *Day 1 Acquisition Phase*: Mice baseline behavior were recorded every 10 s for 120 s acclimation period, followed by a stimulus block which included: (1) a tone for 30 s in duration, (2) 10 s following the tone presentation a light was activated for 10 s, (3) during the last 2 s of tone presentation a 0.5 mA foot shock was presented for 5 s in duration. After the stimulus block another 120 s time period was analyzed every 10 s followed by a 70 s inter-trial-interval (ITI). Four trials were presented during day 1. (b) *Day 2 Retention Phase*: Test procedures were identical to day 1 with the exception of no foot shock and following the stimulus block data were recorded every 10 s for 300 s. (c) *Day 3 Altered Context and Auditory Cued Conditioning Phase*: The same behavioral apparatus was modified with a rubber floor covering the shock grid, a diagonal divider was used, the chamber was black and a novel odorant was placed in the inaccessible portion of the chamber behind the divider to create a triangular altered context. freezing behavior was recorded every 10 s for 180 s followed by the activation of a tone and the same measurements were analyzed. The tone lasted 180 s in duration for the auditory cue.

2.3 Synaptosomal Neurotransmitter Release and Uptake Sample Preparation

Mice were anesthetized with Nembutal® (i.p. injection 50 mg/kg) and sacrificed in accordance with The College of Staten Island's (CUNY) IACUC policies and procedures. In two sets of experiments either their frontal cortices or hippocampi were dissected, extracted, weighed, and homogenized in a 1:10 dilution (mg/mL) buffer containing 0.32 M sucrose, 1 mM $MgSO_4$, 20 mM HEPES and pH adjusted to 7.4 with NaOH (Sigma/Aldrich, MO). Samples were pestled in a glass homogenizer with 18 strokes and 1 mL of the sample was collected then centrifuged at 5,000 rpm × 10 min at 4 °C (Beckman TL-100 Ultra Centrifuge, MD). The supernatant was collected and re-centrifugated at 19,000 rpm × 20 min at 4 °C, while the pellet was discarded. The subsequent pellet containing synaptosomes was re-suspended in 800 μL of oxygenated (O_2 95 %/CO_2 5 %) aCSF solution containing 124 mM NaCl, 2 mM $CaCl_2$, 4.5 mM KCl, 1 mM $MgCl_2$, 26 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, and 10 mM $C_6H_{12}O_6$ with pH 7.4. (Sigma/Aldrich, MO) then centrifugated for another 2 min at room temperature (Beckman # 348750 Microfuge, CA). This step was repeated twice. The remaining synaptosomal pellet was dissolved by pipette in 1 mL oxygenated aCSF solution. Lastly, 200 μL of the synaptosomes were used in the experiments that follow.

2.4 Synaptosomal Neurotransmitter Release Experiments

Synaptosomal neurotransmitter release samples (i.e. 200 μL) were loaded into an electrically inducible chamber (Brandel SF-12 Suprafusion System, MD) along with 3 μL of [2,2 ^{3}H]-Taurine (Perkin Elmer, Inc., MA); a $GABA_{AR}$ agonist used to regulate calcium homeostasis (El Idrissi and Trenkner 1999), counteract glutamate

induced neuronal excitability, and to prevent epilepsy (El Idrissi and L'Amoreaux 2008; El Idrissi et al. 2005; El Idrissi and Trenkner 2004; El Idrissi et al. 2003). Samples were incubated for 1 h while being temperature regulated at 37–39 °C. Perfusion flow rates were adjusted to 90 % pumping rate (2.25 mL/min). After the incubation period, a 30 min pre-collection period was done to wash out excess background radioactivity that was not uptaken into the synaptosomes. The experimental samples were collected every 2 min in individual scintillation vials. The first nine scintillation vials were averaged and taken as a baseline measure of spontaneous release. Sample 10 was then electrically stimulated with a 10 Hz, 10 mA stimulus for 20 s (Brandel 12 channel electric stimulation unit, MD) and another ten scintillation vials were collected post stimulation. These samples were taken as measurements of evoked release. Post experiment, 3.4 mL of ready safe scintillation cocktail liquid (Beckman Coulter™, CA) were added to all collection vials capped and vortexed for 10 s. All scintillation vials were set in trays and scintillation counts per minute (CPM) were collected for 10 min per vial (Beckman Coulter™ LS 6500 Multipurpose Scintillation Counter, CA).

2.5 Synaptosomal Protein Determination Procedures

The sample tubes from each of the experiments that were prepared for the synaptosomal experiments were used to quantify the protein concentration (μg/μL) per 1:10 dilution of sample (mg/mL). Proteins were ran in triplicates and subjected to a Bradford assay using bovine serum as the reference protein with a Bio-Rad DC Protein colorimetric assay kit (BIO-RAD 500-0116, CA) according to manufacture guidelines. Protein absorbance's were read and data computed using SoftMax Pro® data analysis software from a Spectra Max 340PC microplate reader (Molecular Devices, CA). Synaptosomal counts per minute (CPM) of [2,2 3^{H}]-Taurine were recorded over a 10 min period per sample using a Beckman Coulter LS6500 Multipurpose Scintillation Counter (Fullerton, CA). Obtained values for [2,2 3^{H}]-Taurine CPM data were taken and divided by the amount of protein from each respective 1 mL sample. The resultant CPM/mg protein was graphed as amount of basal uptake and spontaneous release rates of [2,2 3^{H}]-Taurine prior to and post electrical stimulation. Results were normalized against the first nine baseline scintillation vials prior to electrical stimulation to determine the relative difference in [2,2 3^{H}]-Taurine release from synaptosomes. Here forth, [2,2 3^{H}]-Taurine will be referred to as ^{3H}-Taurine.

2.6 Statistic Analysis

Statistical significance was determined by *ANOVA* with Tukey's HSD post hoc test. Each value was expressed as the mean ± SEM. Differences were considered statistically significant when ($p < 0.05$).

3 Results

3.1 KO Mice Exhibit Delayed Fear Acquisition Learning When Compared to WT Mice

In this study we examined the effects of acute or chronic taurine supplementation on WT and KO mice emotional learning and memory. We first characterized whether there were any learning deficits in context fear learning between the genotypes under investigation. Figure 1 shows that WT mice showed stable and increasing fear learning as a function of trials (p<0.001***), whereas KO mice exhibited learning deficits until the fourth trail when compared to the mice KO baseline (p<0.05#) and significant emotional learning deficits when compared to WT mice at each trial (p<0.001###). This suggests that KO mice can acquire fear learning but have a delayed acquisition rate for this emotional memory formation. Figure 2 shows that when WT mice are exposed to a-Tau and c-Tau that they initially exhibit increased sensitivity to fear learning when compared to controls (p<0.001***). Following fear acquisition training a-Tau mice exhibit a reduction in fear learning when compared to controls (p<0.001†††), whereas c-Tau mice show increased sensitivity to fear learning (p<0.001###). Moreover, WT mice may have a difficulty with fear

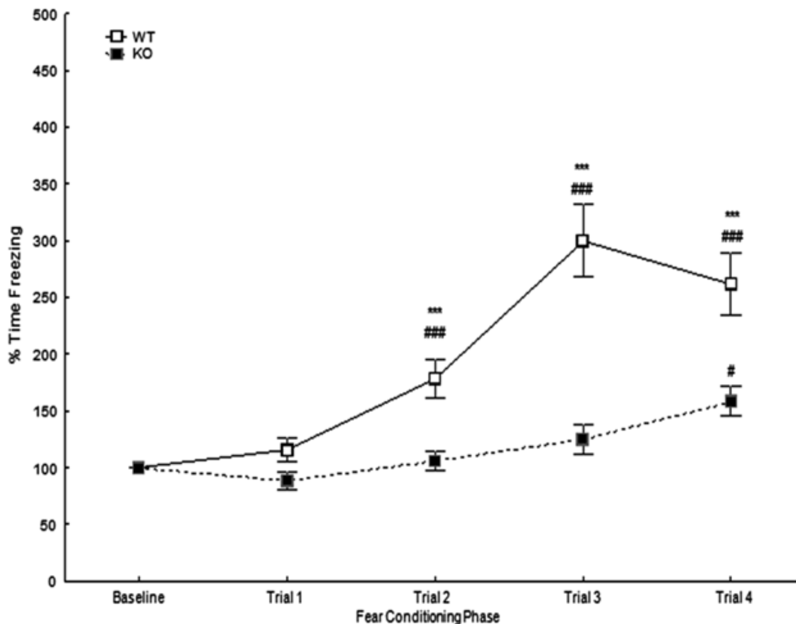

Fig. 1 Genotype comparisons in context fear acquisition learning. WT mice showed increased fear learning as a function of training (p<0.001***), whereas KO mice exhibited delays in fear learning at trial 4 (p<0.05#). When comparing genotype effects WT mice evidenced intact fear learning traits in contrast to learning deficits in the KO mice across all trials (p<0.01###)

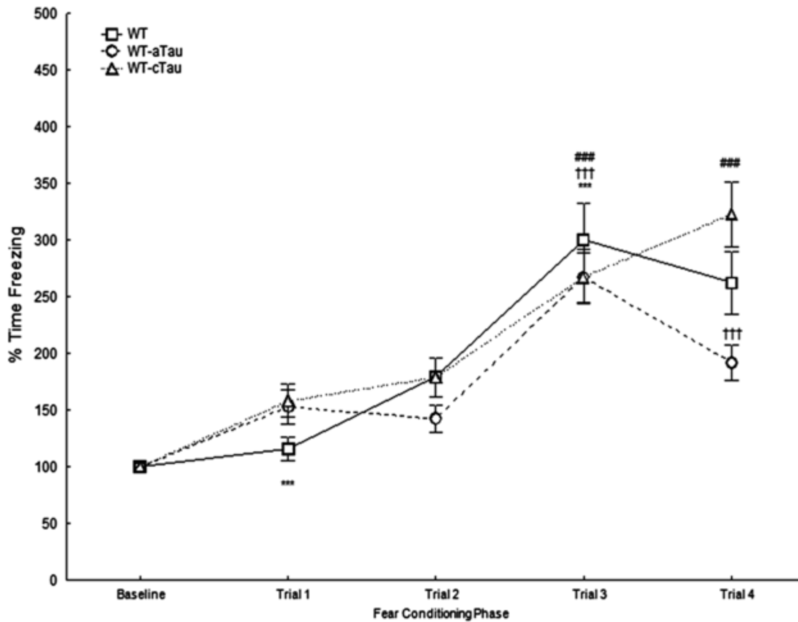

Fig. 2 Effects of a-Tau and c-Tau on WT fear learning. Mice treated with taurine on trial 1 show increased fear responsivity when compared to control mice ($p < 0.001$***). At trial 2 taurine shows differential effects on WT fear learning and by trial 4 a-Tau treatment results in decreased fear learning when compared to WT mice ($p < 0.001$†††), whereas c-Tau treatment increased fear learning when compared to WT ($p < 0.001$###). All treatment conditions resulted in fear learning from baseline ($p < 0.001$***, †††###). This suggests that WT mice may have a difficulty with fear learning as a decline in cognitive functioning as a consequence of age and that taurine exposure may facilitate modifications in emotional learning and memory based on exposure type (re-printed with permissions from Springer Press; Neuwirth et al. 2013)

learning as a decline in cognitive functioning as a consequence of age and that taurine exposure may facilitate modifications in emotional learning and memory based on exposure type. Figure 3 shows that KO mice were extremely sensitive to a-Tau and c-Tau treatment more than WT mice. In KO mice taurine differentially affected emotional fear learning outcomes. KO mice had significant delays in fear learning until trial 4 ($p < 0.05$*), whereas a-Tau and c-Tau significantly increased fear learning when compared to KO mice ($p < 0.001$†††, ###). Interestingly, c-Tau recovered KO mice learning deficits to levels comparable to WT mice with stable learning performances ($p < 0.001$###). In contrast, a-Tau induced hyper sensitivity to fear learning when compared to KO mice ($p < 0.001$†††, †††). This suggests that c-Tau supplementation may positively modify $GABA_{AR}$ expression levels under c-Tau treatment and a-Tau produces hyper sensitivity to emotional based stimuli which may negatively impact emotional learning and memory.

Following context fear learning we assessed mice emotional memory retention as a function of genotype and taurine supplementation in a 24 h re-test without shock. Our findings show in Fig. 4 at 24 h following training all mice retain fear memories

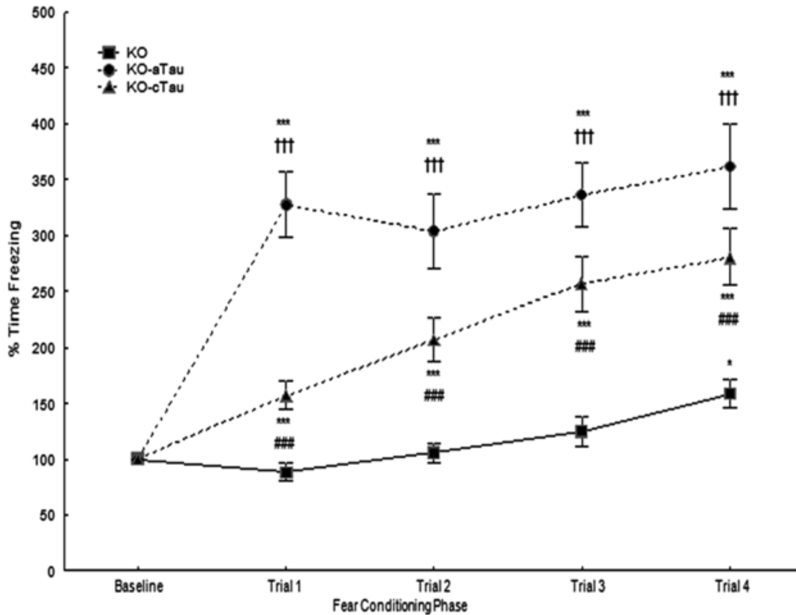

Fig. 3 Effects of a-Tau and c-Tau on KO fear learning. KO mice exhibit learning deficits until trial 4 ($p<0.05$*). c-Tau restores fear learning to levels comparable to WT mice ($p<0.001$###), whereas a-Tau enhances sensitivity to levels exceeding the typical range of WT mice ($p<0.001$†††). This suggests that c-Tau supplementation may positively modify $GABA_{AR}$ expression levels under c-Tau treatment and a-Tau produces hyper sensitivity to emotional based stimuli which may negatively impact emotional learning and memory. a-Tau and c-Tau treatments differentially affected KO mice emotional learning and memory and were significantly different from KO mice across trials ($p<0.001$†††, ###)

when compared to the last trial during the acquisition phase. However, KO mice have the least amount of learned fear during training and the least degree of change in memory retention. WT mice have a substantial reduction in fear retention 24 h post training ($p<0.001$***). Notably, in WT a-Tau mice memory acquisition was reduced when compared to WT mice and 24 h later their memory was stabilized with little loss in retention, whereas in WT c-Tau mice treatment resulted in enhanced fear learning, but significant retention loss ($p<0.001$, ###). In KO mice a-Tau and c-Tau increased memory acquisition ($p<0.001$†††, ###), but 24 h later only a-Tau stabilized memory retention ($p<0.001$, †††). Interestingly, 24 h post training KO-c-Tau memory returned to levels comparable to KO mice. This suggests that in WT mice c-Tau supplementation enhances emotional memory acquisition and retention in aged mice, whereas a-Tau produces the same phenomenon in aged KO mice.

During baseline in the altered context WT and KO mice explore the novel context more than all other treatment groups despite age related effects (Fig. 4). KO-a-Tau exhibit elevated freezing behavior when compared to KO ($p<0.001$, †††). When the learned auditory cue is presented in the altered context WT a-Tau and

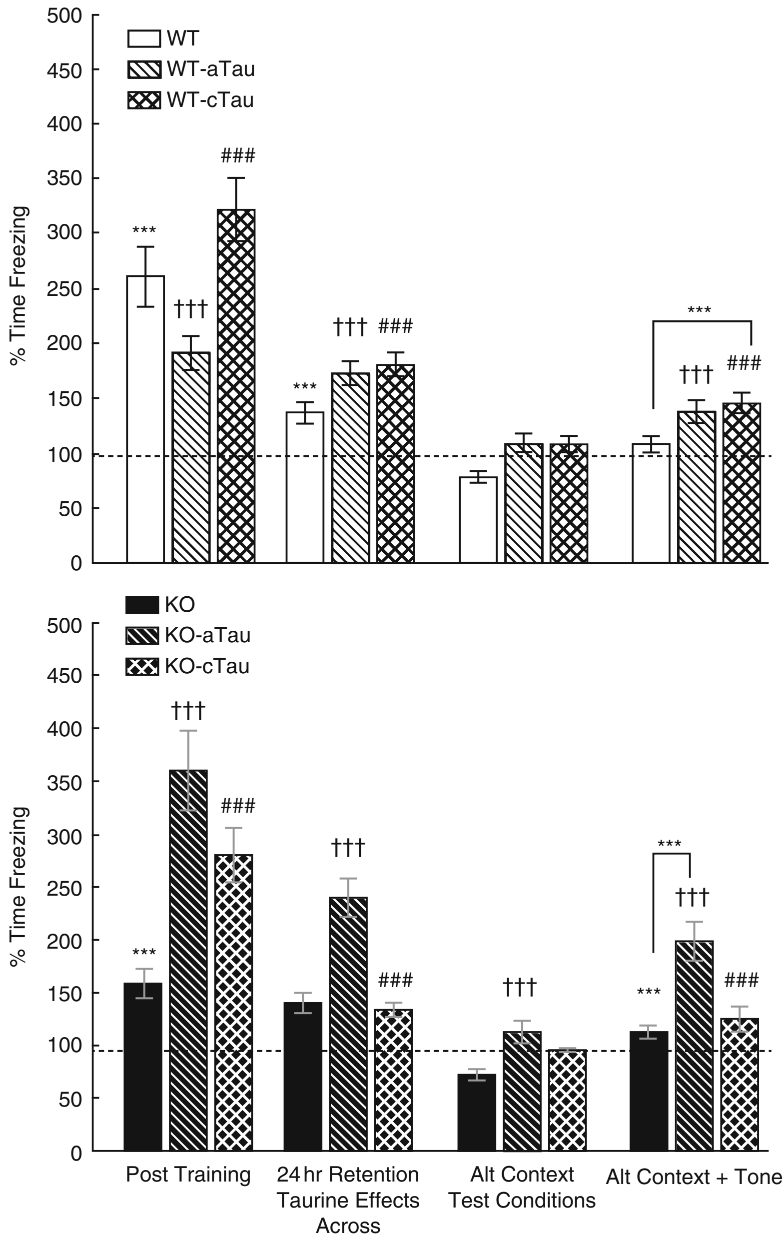

Fig. 4 Genotype and taurine effects across context fear and auditory cued conditioning learning and retention in aged mice. *Horizontal dotted line* represents baseline level in freezing behavior for each respective genotype. Data show at 24 h all mice retain the fear memory, however in WT mice both a-Tau reduces memory learning and c-Tau enhances memory ($p<0.001$, †††, ###) when compared to WT mice; whereas, in KO mice a-Tau and c-Tau increased memory acquisition ($p<0.001$, †††), but retention was only stabilized in a-Tau mice ($p<0.001$, †††) when compared to KO mice. During baseline in the altered context KO-aTau shows elevated freezing behavior when compared to KO mice ($p<0.001$, †††). When the learned auditory cue is presented in the altered context in WT mice a-Tau and c-Tau show increased freezing behaviors ($p<0.001$, †††, ###) when compared to WT mice; contrastingly, in KO mice on a-Tau show enhanced freezing ($p<0.001$, †††). This suggests taurine has differential effects on mice genotype which alters fear memory neurochemically based on acute or chronic exposure

c-Tau mice show increased freezing behaviors ($p<0.001$, †††, ###) when compared to WT mice; whereas, in contrast only KO a-Tau mice show enhanced freezing ($p<0.001$, †††). This suggests that KO a-Tau mice are hypersensitive to novel environments and sensory input producing elevated freezing responses. Thus taurine differentially regulates GABAergic mediated fear signaling in these mice as a function of taurine exposure and genotype.

3.2 Cortical Synaptosomes Contribute Greater Taurine Neurochemical Signaling in Regulating Fear Learning When Compared to Hippocampal Synaptosomes with Enhanced Neurophysiological Properties in KO Mice

To further explore how taurine differentially mediates fear learning and memory in the WT and KO mice we sacrificed animals immediately following testing to evaluate the neurochemical signals within frontal cortex and hippocampus. The frontal cortex is critical for attentional-based mechanisms, goal directed behavior, and planning motor commands whereas the hippocampus is responsible for integrating the multi-sensory experiences of the animal during testing to form a memory trace of the learned experience for later recall and more efficient action planning in future experiences. Thus, we prepared synaptosomes (i.e. nerve terminal ends from these brain structures) and subjected them to neurophysiological assays to evaluate ^{3H}-taurine uptake and release (Figs. 5 and 6). In non-stimulated frontal cortex synaptosomes c-Tau increased ^{3H}-taurine-uptake and spontaneous release in both WT ($p<0.01$**) and KO mice ($p<0.001$###) (Fig. 5). Notably, KO c-Tau mice significantly accumulated and released more ^{3H}-taurine than WT mice prior to stimulation. This suggests aberrant non-specific firing of taurine at a basal state in the frontal cortex of KO c-Tau mice. Following stimulation, WT c-Tau mice released more ^{3H} -taurine than WT mice ($p<0.001$***) and similarly KO c-Tau mice revealed the same phenomena when compared to KO mice ($p<0.001$###) (Fig. 5). Post stimulation KO c-Tau mice showed increased ^{3H}-taurine release when compared to WT mice suggesting that this enhanced taurine release resulted from the learned fear related and stress induced behavioral states and serves a neuroprotective event. Our data revealed that frontal cortical synaptosomes (Fig. 5) evidenced a *Condition* effect $F(3,15)=4.58$, $p=<0.05$*; a *Genotype* effect $F(3,15)=91.10$, $p<0.001$***; a *Treatment* effect $F(3,15)=88.42$, $p<0.001$***; a *Condition X Genotype* interaction $F(3,15)=27.20$, $p<0.001$***; and a *Genotype X Treatment* interaction $F(3,15)= 12.81$, $p<0.001$***.

In hippocampal synaptosomes, taurine treatment enhanced both WT ($p<0.01$**) and KO mice ^{3H}-taurine uptake and release in non-stimulated synaptosomes identical to the observations seen in frontal cortex (Fig. 6). Similarly, KO c-Tau mice exhibited significant uptake and release of 3H-taurine when compared to WT mice at basal states ($p<0.001$***). Post stimulation, taurine enhanced ^{3H}-taurine release from WT c-Tau and KO c-Tau mice, whereas WT and KO mice exhibited similar

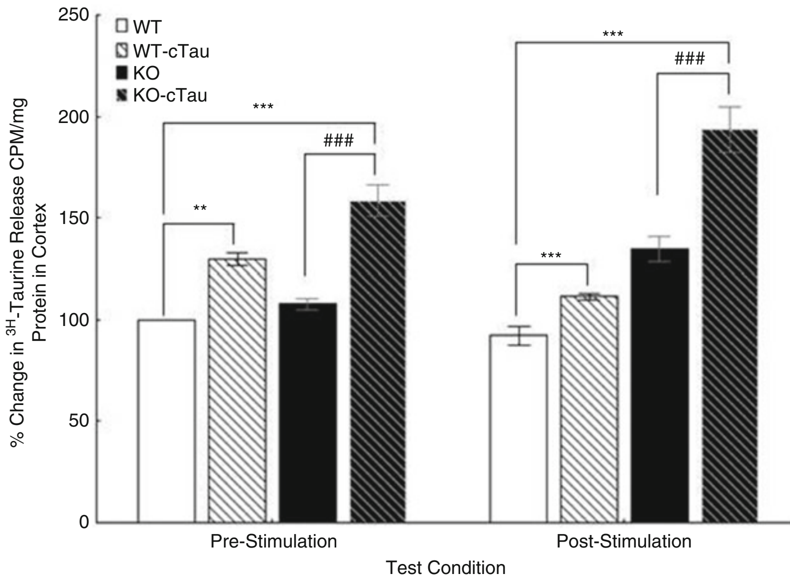

Fig. 5 Genotype and ^{3H}-taurine effects in frontal cortical synaptosomes following fear and auditory cued conditioning learning in aged mice. Pre-stimulation depicts basal levels of ^{3H}-taurine uptake into FCX synaptosomes. Post-stimulation shows the electrically induced release of ^{3H}-taurine from FCX synaptosomes. ^{3H}-taurine uptake into FCX synaptosomes is increased in WT-cTau and KO-cTau mice at baseline in contrast to WT and KO mice ($p<0.01$**, $p<0.001$###) respectively. Following electrical stimulation WT-cTau ($p<0.001$***), KO, and KO-cTau mice ($p<0.001$###) showed enhanced ^{3H}-taurine release when compared to WT. Notably, KO-cTau mice ($p<0.001$###) exhibited the largest storage and release of ^{3H}-taurine in contrast to all other groups. Suggesting, that KO mice have increased cortical sensitivity to taurine and have additional modulation of GABAergic signaling during fear learning selective to KO mice

events. Data from hippocampal synaptosomes (Fig. 6) evidenced a *Condition* effect $F(3,15)=7.55$, $p<0.006$**; a *Treatment* effect $F(3,15)=33.09$, $p<0.001$***; and a *Condition X Treatment* interaction $F(3,15)=5.37$, $p<0.02$*.

Taken together these neurophysiological data suggest that taurine during basal states may mediate neurochemical signals to attenuate increased hyper-excitable states as a neuroprotective mechanism to increase the efficiency of GABAergic information processing within and amongst the frontal cortex and hippocampus to facilitate emotional learning and memory.

4 Discussion

Our study investigated the effects of taurine on age, emotional learning and memory as a function of genotype and exposure method. Consistent with our previous findings (El Idrissi et al. 2012; Neuwirth et al. 2013), taurine produced two opposing

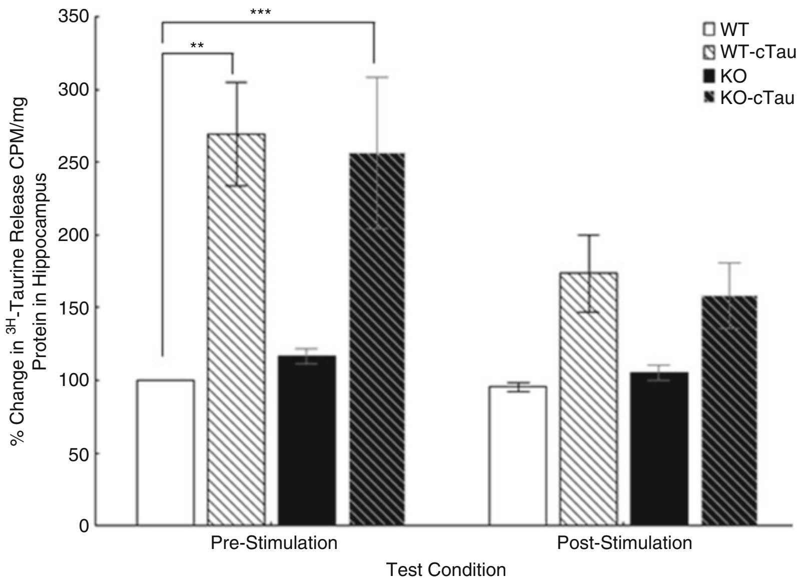

Fig. 6 Genotype and 3H-taurine effects in hippocampal synaptosomes following fear and auditory cued conditioning learning in aged mice. Pre-stimulation depicts basal levels of 3H-taurine uptake into HP synaptosomes. Post-stimulation shows the electrically induced release of 3H-taurine from HP synaptosomes. 3H-taurine uptake into HP synaptosomes is increased in WT-cTau and KO-cTau mice at baseline in contrast to WT and KO mice ($p<0.01$**, $p<0.001$###) respectively. Following electrical stimulation WT-cTau and KO-cTau showed enhanced 3H-taurine release when compared to WT and KO, but these data were not significant. This suggests that in hippocampus 3H-taurine uptake and release is enhanced by taurine treatment contributing to fear learning and memory irrespective of genotype

phenotypes: an anxiolytic behavioral signature in response to a-Tau and an anxiogenic signature in response to c-Tau in WT mice. Notably, in KO mice due to a reductions in $GABA_{AR}$ expression (El Idrissi et al. 2005), gene expression changes in $GABA_{AR}$ subunits (Shen et al. 2013), genetically induced state of hyperactivity/hyper-excitability (El Idrissi et al. 2010) and compromised intellectual capacity (El Idrissi et al. 2013) we observed a reversal in pharmacological action of taurine in these behavioral signatures when compared to WT mice consistent with our prior studies (El Idrissi et al. 2009, 2009; El Idrissi 2008). Thus, a-Tau produces anxiogenic behavioral signatures, whereas c-Tau produces anxiolytic behavioral signatures in KO mice. To this end, this suggests that c-Tau in KO mice provides stress induced neuroprotection from brain excitability consistent with others reports (Riback et al. 1993; Wu et al. 1998; Suge et al. 2007), that may be further modified by behavioral experience such as fear induced learning and memory may be further explored as a potential pharmacotherapy for treating aged Fragile X mice.

Post testing, the synaptosomal preparations from these animals showed an enhanced uptake and release of 3H-taurine in non-stimulated conditions suggesting enhanced non-specific firing of taurine to counteract brain over-excitability induced

by stressful fear learning. When stimulated the taurine treated animals evinced enhanced ^{3H}-taurine release from WT c-Tau and KO c-Tau mice, whereas WT and KO mice exhibited similar events. These findings were more pronounced in the frontal cortex than hippocampus suggesting taurine to afford greater neuroprotection directed towards the attentional-based working memory mechanisms more so in this learning task in aged mice. This observation may be specific to aged animals and different findings may be observed in young mice. This would explain why in the aged WT a-Tau mice and KO c-Tau mice exhibited enhanced fear acquisition and subsequently 24 h later a near erasure of emotional fear learning. Taken together these neurophysiological data suggest that taurine during basal states may mediate neurochemical signals to attenuate increased brain hyper-excitability as a stress-induced neuroprotective mechanism to increase the efficiency of GABAergic information processing within and amongst the frontal cortex and hippocampus to facilitate emotional learning and memory.

5 Conclusion

In summary, this study showed that taurine differentially affects aged mice based on genotype and taurine exposure with more pronounced effects on taurine induced GABAergic signaling in the frontal cortex following fear learning. This suggests that taurine exposure may attenuate stress-induced brain excitability brought forth through behavioral learning as a neuroprotective mechanism thereby increasing the efficiency of GABAergic neurochemical signaling that regulates attentional-based and memory relevant information during active emotional states in aged mice. Thus, taurine may present as a potential pharmacotherapuetic intervention for aged Fragile X mice in recovering emotional states, such as anxiety and stress in the context fear and auditory cued conditioning test paradigms with potential to be further generalized across other emotional learning and memory tests..

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Contribution of Taurine Signatures in the Detached Cat Retina

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Abbreviations

RD Retinal detachment
Tau Taurine

1 Introduction

Several studies have investigated the histopathology of the detached cat retina (Anderson et al. 1983; Erickson et al. 1983). In these studies, the photoreceptor outer segments begins to show distortion and vacuolization in the distal tips 12 h after detachment and show evidence of disruption between 24 and 72 h after detachment (Anderson et al. 1983). The photoreceptor inner segments begin to change. Some inner segment organelles including mitochondria are swollen and the

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number of organelles decrease between 24 and 72 h after detachment. The outer nuclear layer begins to show signs of necrosis, including distended mitochondria and endoplamic reticulum in perikarya at 3 days after detachment (Erickson et al. 1983). In the outer plexiform layer, the rate of change due to detachment is more rapid. Terminals of the photoreceptor especially rod spherules show vacuolization at 1 h after detachment (Erickson et al. 1983). These rapid changes in the photoreceptor cells are followed by a significant decrease in the number of photoreceptor nuclei. Photoreceptor cells, which are sensitive to retinal detachment, may show different amino acid localizations from those that are resistant to the insult because some amino acids are strongly involved in energy metabolism. Especially, the formation and degradation of both glutamate and γ-aminobutyric acid GABA are intimately coupled with each other and the citric acid cycle and hence very likely to be affected changes in oxygen availability (Robin and Kalloniatis 1992; Kalloniatis and Napper 1996; Marc et al. 1998a). It is crucial to clarify the relationship between taurine distribution and its histopathological change in the detached retinas. Previous immunocytochemical studies have demonstrated the localization of several amino acids in the cat retina or the detached vertebrate retina (Marc et al. 1998a; Sherry and Townes-Anderson 2000; Marc et al. 1998b). The enucleated eyes were fixed after death by immersion in all cases (Marc et al. 1998a, b; Chun and Wassle 1989; Jojich and Pourcho 1996). We have shown that photoreceptor inner segments in the cat retina have weak glutamate immunoreactivity in contrast to the results of previous studies (Sasoh et al. 1998) and that perfusion fixation is critically important for prevention of postmortem artifact when studying the immunochemical distribution of amino acids in the retina (Takeo-Goto et al. 2002). Thus, we examined immunocytochemical labelling taurine following perfusion fixation. The present study investigates the distribution of taurine in the detached cat retina with minimum postmortem artifact and attempts to clarify the relationship between taurine and observe its localization changes in the outer portion of detached retinas.

2 Materials and Methods

2.1 Animal and Experimental Design

Eyes from 16 cats (around 3.0 kg in weight) with unilateral rhegmatogenous retinal detachment were fixed at 15, 60 min, 12, 24, 48, 72, 168 h after detachment. We used two animals per time point, and the contralateral eyes served as controls. All animals were maintained on a 12-h light; 12-h dark cycle throughout the course of the study. The experiments adhered to the Guiding Principles in the Care and Use of Animals.

2.2 Surgical Procedure

The operative procedure was essentially the same as described (Sasoh et al. 1998; Geller et al. 1995). Briefly, cats were anesthetized with an intramuscular injection of 20 mg/kg ketamine hydrochloride (Ketalar, 50 mg/ml, Sankyou, Tokyo, Japan) and 20 mg/kg sodium pentobarbital (Nembutal, 40 mg/ml, Abbott laboratories, North Chicago, USA). Then, a conjunctive injection of 0.5 ml of 2 % xylocaine was given. First, the lens was removed by a phacoemulsification technique. Two weeks later, a small glass pipette (external tip diameter 80–100 μm) was inserted between the neural retina and the retinal pigment epithelium monolayer, and 0.25 % sodium hyaluronate (Opegan Hi, 10 mg/ml Seikagaku Corporation, Tokyo, Japan) in a balanced saline solution was then infused to separate the retina from the retinal pigment epithelium. Animals were fixed by intracardiac perfusion of 1 % glutaraldehyde and 4 % paraformaldehyde in 100 mM sodium phosphate buffer (PBS) (pH 7.4) including 2 % sucrose. After perfusion, the eyes were enucleated, the anteriour one-third of the globes were excised and the eye cups were immersed overnight in the same fixative mixture. Then they were rinsed several times with 50 mM Tris–HCl buffer, pH 7.4, dehydrated with a graded alcohol series and embedded in paraffin. Five-micrometer thick sections were mounted on albumin-coated slides.

2.3 Antibodies

Taurine specific antibody was prepared in our laboratory. In brief, taurine was coupled with rabbit serum albumin (RSA) via glutaraldehyde and taurine-GAL-RSA conjugate, was emulsified with an equal volume of complete Freund's adjuvant and repeatedly injected intracutaneously into multiple sites on the back of two rabbits. Taurine antibody was purified by affinity chromatography with taurine immobilized on cellulofine GCL-2000m, and specificity of this antibody was examined by a dot immunobinding assay (Ma et al. 1994; Ma et al. 2003). The antibody demonstrated a significant reactivity only with taurine. To confirm immunoreactivity of this antibody in histological sections, rat cerebellar sections were studied with this antibody. As in previous studies, Purikinje cells and their dendrites exhibited intense immunostaining (Ma et al. 2003).

2.4 Immunohistochemical Study

Retinal sections were deparaffinized in xylene and rehydrated through a graded alcohol series and washed twice for 5 min in PBS. They were left in 0.3 % H_2O_2 in methanol for 30 min. Sections were incubated in normal goat serum that had been

diluted 1:50 in PBS. The sections were incubated with purified taurine antibody (0.1 μg/ml PBS) for 16 h, and for 2 h with antibodies against rabbit IgG that had been raised in goat (diluted 1:200 in PBS). After 60 min incubation with the peroxidase-anti-peroxidase complex, they were washed in PBS. Finally, the sections were incubated with a solution that contained 3′-diaminobenzidine tetrahydrochloride (DAB) as chromogen. The stained sections were examined using Olympus BX53 microscopy (Olympus Tokyo, Japan).

3 Results

Figure 1 shows taurine staining in the normal retina fixed by perfusion. Taurine immunoreactivity was observed in many ganglion cells. The inner plexiform layer also presented taurine immunoreactivity. However, the inner and outer segments of photoreceptors and the outer nuclear layer did not seem to be taurine immunoreactive (Fig. 1 Left). Negative staining was preformed with non-immune serum used instead of the taurine antibody and revealed no positive staining (Fig. 1 Right).

Figure 2 shows taurine staining in the retinas after 15 and 60 min detachment. Even if retinal thickness increased as a result of retinal edema after detachment, the profile of taurine presented in the ganglion cells layer and inner plexiform layer became weaken at 15 min, and it increased in the outer plexiform layer significantly at 60 min (Fig. 2). Immunoreactivities of the outer nuclear layer for taurine, which were not detectable in the normal retinas, were already evident by 15 min after detachment. Taurine immunoreactivity was evident at the edge of retinal detachment and gradually increased in proportion to the height of detachment (Fig. 2). The immunoreactivities gradually increased in time intervals in the outer nuclear layer until 24, 48 and 72 h after detachment. Retinas 168 h after detachment showed weak and somewhat diffuse staining for taurine (Fig. 3).

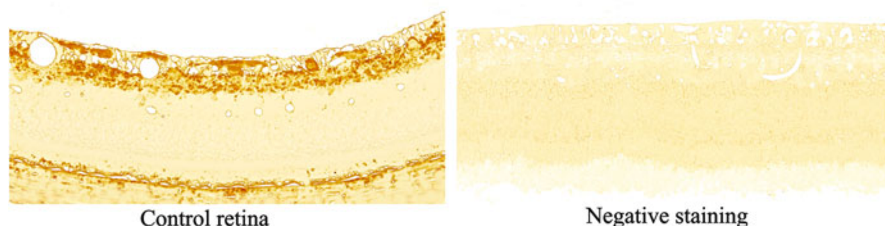

Fig. 1 Distribution of taurine-specific immunoreactivity in the normal cat retina. Taurine immunoreactivities of the ganglion cell layer and inner plexiform layer were observed in the normal cat retina. No immunoreactivity was observed in the negative staining without primary taurine antibody

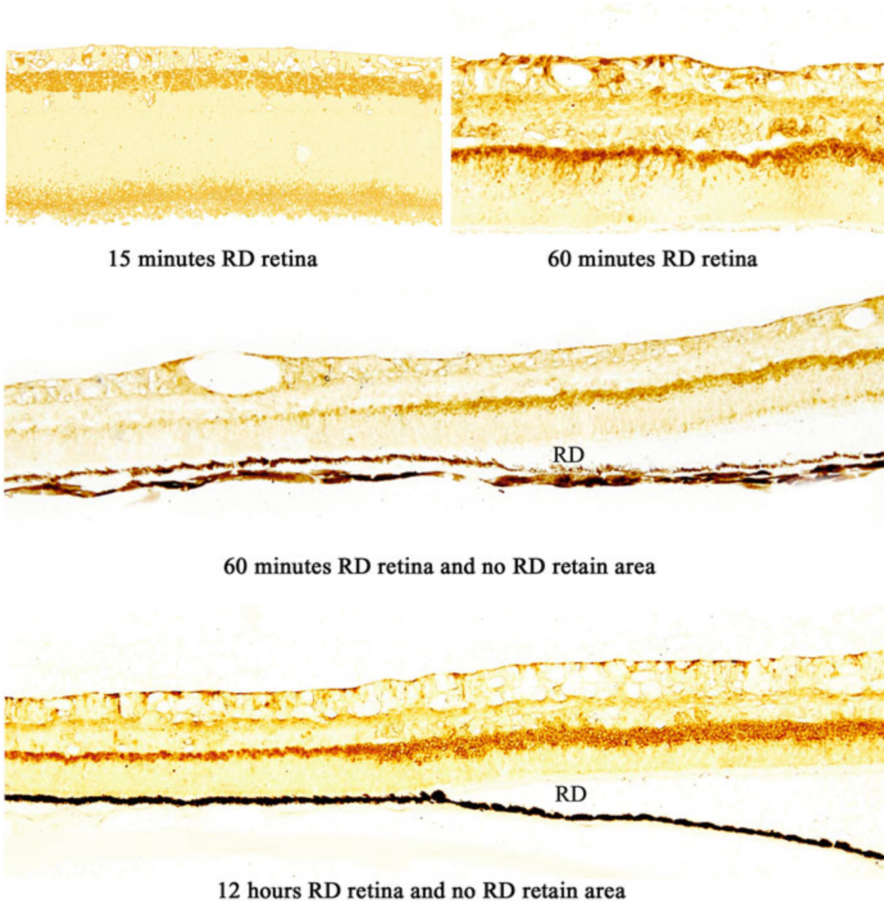

Fig. 2 Distribution of taurine-specific immunoreactivity in the 15 and 60 min after retinal detachment. Taurine immunoreactivities decreased in the ganglion cell layer and inner plexiform layer at 15 min, and in the outer plexiform layer increased significantly at 60 min. At 60 min after retinal detachment, the taurine immunoreactivity was increased in the outer nuclear layer and weak immunoreactivity was observed in the photoreceptor layer. At 12 h after retinal detachment, intense taurine immunoreactivity was observed in the outer plexiform layer. *RD* retinal detachment area

4 Discussion

Taurine is a semi-essential amino acid and is not incorporated into proteins (Schuller-Levis and Park 2003). In mammalian tissues, taurine is ubiquitous and is the most abundant free amino acid in the retina (Ripps and Shen 2012). In fact, taurine is tissue-protective in many models of oxidant-induced injury (Keys and Zimmerman 1999). One possibility is that taurine reacts with hypochlorous acid, produced by

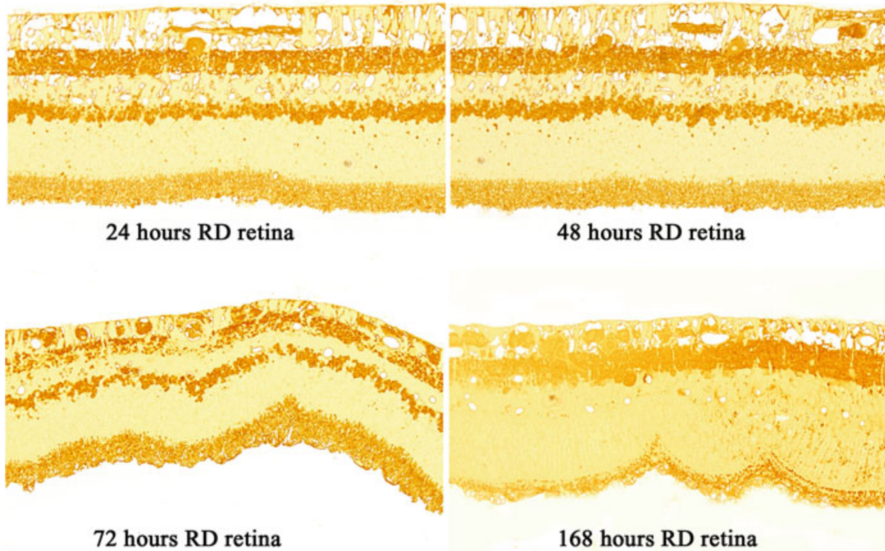

Fig. 3 Distribution of taurine-specific immunoreactivity in the 24, 48, 72 and 168 h after retinal detachment. Taurine immunoreactivities increase in the inner plexiform layer (IPL), outer plexiform layer and photoreceptor layer from 24 to 72 h. Taurine immunoreactivity in the outer plexiform layer and photoreceptor layer decreased at 168 h

the myeloperoxidase pathway, to produce the more stable but less toxic taurine chloramine (Tau-Cl) (Schuller-Levis and Park 2003; Chorazy et al. 2002). Data from several laboratories demonstrate that Tau-Cl is a powerful regulator of inflammation. Taurine transporter knockout mice show reduced taurine, reduced fertility, and loss of vision due to severe apoptotic retinal degeneration (Heller-Stilb et al. 2002). Oxygen supply in the outer avascular half of the retina is dependent primarily on diffusion from the choroidal circulation. Oxygen tension of the outer retina, especially the inner segments dramatically decreases during retinal detachment (Linsenmeier and Padnick-Silver 2000). Although diffusion of other substances and metabolites would also certainly be compromised following retinal detachment, oxygen plays a key role in the preservation of photoreceptors (Mervin and Stone 2002a, b). These studies suggest that the outer retinas, especially the inner segments, enter into anoxic or hypoxic states during detachment because the inner segments have a high oxygen demand. Thus, not surprisingly striking changes in taurine distribution showed a concentration in the outer retina at the early stage of detachment especially.

The present study exclusively focused on the outer portion of the retina for this reason in part. To date, two types of photoreceptor cell death after retinal detachment have been reported: necrosis (Erickson et al. 1983) and apoptosis (Hisatomi et al. 2001; Hisatomi et al. 2002). In our previous study, we demonstrated that the necrotic photoreceptor cell, which is characterized by distension of cytoplasm,

especially mitochondria, tends to cluster. The apoptotic photoreceptor cell, which is characterized by chromatin condensation and cell shrinkage, always appears isolated from each other. The majority of the damaged photoreceptor cells had histopathological features of necrosis (Sasoh et al. 1998). We observed the relationship between the changes of taurine distribution and apoptosis in the same retinal layer after retinal detachment. From the present results about taurine distribution of the photoreceptor cells, it is quite possible that excess levels of taurine in the cytoplasm of photoreceptor cells preceded anti-oxidant effects in the detached retinas. But it is still unclear whether excess of taurine in the outer segment of photoreceptor layer.

The present results and previous studies (Mervin et al. 1999; Linsenmeier and Padnick-Silver 2000) strongly suggest that histopathological and immunocytochemical changes in the photoreceptor cells are the consequence of ischemia or hypoxia due to retinal detachment. Taurine is an inhibitory amino acid, which has osmoregulatory, neuromodulatory and possibly neuroprotective effects in neuronal tissue (Shuaib 2003; Schurr et al. 1987). Photoreceptor cells having the excess taurine in their cytoplasm after detachment is utilized to resist intracellular hypo-osmolality and maintain a normal intracellular balance (Saransaari and Oja 2010). The previous studies reported that the intracellular concentration of glutamate, aspartate and glutamine decrease in neural cells and glutamate uptake is disrupted following brain ischemia (Robin and Kalloniatis 1992). The present results in retinal detachment are in contrast to the previous results in brain tissue. Thus, the explanation for why taurine simultaneously increase in the photoreceptor cells including photoreceptor terminals of detached retinas remains unclear. The present study has an important limitation, and several issues are left for future study. The previous studies have demonstrated that photoreceptor apoptosis plays an important role in the pathophysiology of retinal detachment (Cook et al. 1995; Arroyo et al. 2005; Torp et al. 1993). However, the apoptotic mechanisms were not investigated in the fixed retinal samples of this study.

It is entirely possible that retinal ischemia alters taurine immunoreactivity in the photoreceptor inner segment before the outer retina is sufficiently fixed by glutaraldehyde. Our results are consistent with this because they demonstrate that the inner segment of the detached retina is taurine immunoreactive within 15 min of detachment. We used a mixture of 1 % glutaraldehyde and 4 % formaldehyde as a fixative. Previous studies have generally applied a higher concentration of glutaraldehyde and a lower concentration of formaldehyde. However, Marc and coworkers reported glutamate immunoreactivity at 0.5 % glutaraldehyde concentration. Furthermore, they reported that glutamate immunoreactivity is equally preserved between 0.5 and 2.5 % glutaraldehyde, regardless of formaldehyde content (Marc et al. 1990). Similarly, Aoki and coworkers reported that 1 % glutaraldehyde concentration was effective in diminishing the background-staining intensity (Aoki et al. 1986). Thus, the fixative mixture used in the present study likely did not result in any adverse effects on the preservation of taurine immunoreactivity. In the present study, little or no taurine immunoreactivity was observed in the inner segments of photoreceptors under normal metabolic conditions. Studies of the retina will help further understanding of retinal ischemia because taurine immunoreactivity can be considered a

parameter of the degree of the retinal anoxic condition, including retinal detachment. Furthermore, the effects of therapeutic drugs on ischemia can be estimated using taurine immunoreactivity. Thus, perfusion fixation will be critically important to avoid die artifactual redistribution of taurine.

5 Conclusion

In summary, the present study demonstrates excess intracellular taurine in photoreceptor cells due to retinal detachment followed by necrosis. Our findings strongly suggested that these changes were caused by a disturbance of energy metabolism in the outer retina. These data are discussed relative to taurine's role in the process of rhodopsin regeneration and in the protection of the rod outer segments against osmotic, mechanical and light induced damage.

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Effect of Taurine on Reproductive Hormone Secretion in Female Rats

Teng Mu, Jiancheng Yang, Zhong Li, Gaofeng Wu, and Jianmin Hu

1 Introduction

Taurine (2-aminoethansulfonic acid), first isolated in 1827 from ox-bile, is one of the end products of sulfur metabolism (Tiedemann and Gmelin 1827), is one of the most abundant free amino acid in animal bodies, widely exists in tissues and cells. Secretory organs, such as hypothalamus adenohypophysis and neurohypophysis all contain taurine at higher concentrations (Pow et al. 2002); taurine is also one of the main amino acid secreted by the female reproductive tract (female reproductive tract includes uterus and tubal). The ovaries contain taurine transporter (TauT) RNA (Harris et al. 2005). Oviduct epithelial cells can synthesize taurine through CSAD pathway, then secrete it into the liquid in the infusion tube (Guerin et al. 1995). Taurine has been suggested to promote embryonic growth after *in vitro* fertilization (Barnett and Bavister 1992). Taurine supplementation in chicken forage can accelerate gonad development, improve the level of cock testosterone and hen plasma estradiol (Xiao et al. 1997). During pregnancy and lactation, lack of taurine causes growth stagnation, malformation, retinal degeneration, myocardial damage and

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disorders of central nervous system (Sturman 1993). The ameliorative effect of taurine in the protection against toxemia has been shown during pregnancy in female rats (Ibrahim et al. 2009). Although there are many studies showing that taurine is very essential for reproduction of female animal, no information is available on how taurine regulates the reproduction of female animals.

In the present study we used ELISA to investigate how taurine affects the secretion of six hormones from hypothalamus, pituitary and ovaries during various stages of the estrous cycle.

2 Methods

2.1 Experimental Design

80 purchased female rats were randomly divided into 5 groups, 16 each: blank control group (C), 1 % taurine model group (T1), 2 % taurine model group (T2), 1 % β-alanine model group (β1), 2 % β-alanine model group (β2). After 7 days of adaptive feeding, rats in the blank control group drank normal water, taurine groups drank water containing 1 or 2 % taurine, and β-alanine groups drank water containing 1 or 2 % β-alanine. The experiment was carried out for 2 months and at the end of the experiment rats were sacrificed.

2.2 Measurement of Hormones

The estrous cycle was checked through microscopic examination of vaginal exfoliative cell smears before the rats were sacrificed and the vaginal exfoliocytological characteristics were evaluated. Blood was collected and kept at room temperature for 3 h before centrifuging at 1,500 rpm for15 min to separate the serum. Serum was stored at 40 °C.

The serum content of GnRH, FSH, LH, E2, P and PRL was measured by Enzyme-Linked Immunosorbent Assay (ELISA).

2.3 Statistical Analysis

Data were presented as the mean ± SE and significant differences were determined by Duncan's multiple range test using SPSS 16.0 statistical analysis software. P values less than 0.05 were considered significant.

3 Results

3.1 Results of Hormone Contents in Serum Secreted by the Hypothalamus

As shown in Fig. 1, in metestrus, the level of GnRH in the serum was significantly increased by taurine (Group T2) administration ($p<0.05$); but in Proestrus, Estrus and Diestrus, the level of GnRH showed no significant changes ($p>0.05$) in response to taurine supplementation.

3.2 Results of Hormone Contents in Serum Secreted by the Pituitary Gland

As shown in Fig. 2, in Proestrus, Estrus and Diestrus, the level of PRL in the serum was significantly increased by taurine (Group T2) administration ($p<0.05$); the Group T1 was significantly higher than Group C in Proestrus ($p<0.05$); but in Metestrus, the level of PRL showed no significant changes ($p>0.05$).

As shown in Fig. 3, the level of FSH in the serum was increased by taurine administration; but the increase did not reach significance.

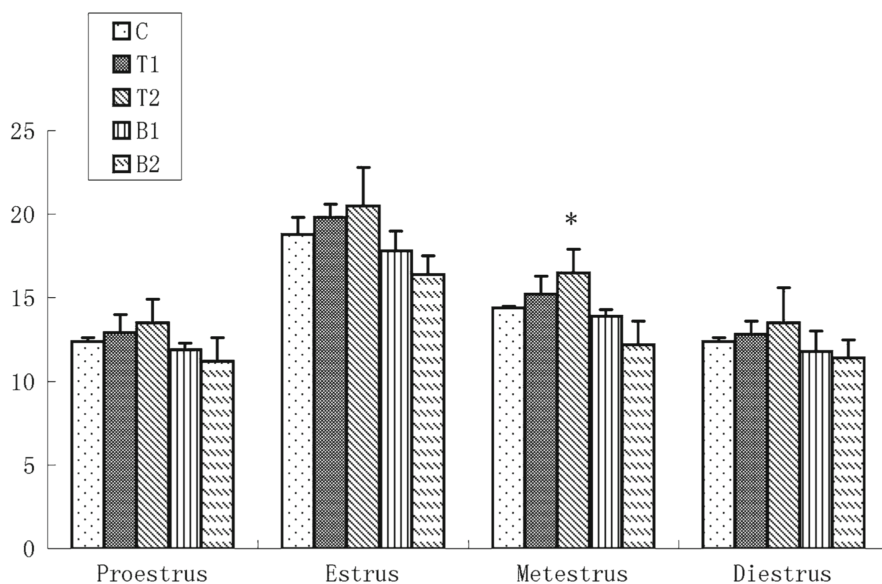

Fig. 1 Effects of taurine on the secretion of GnRH in serum. Results are presented as mean ± SE (n = 6). *Significantly different from control group ($p<0.05$)

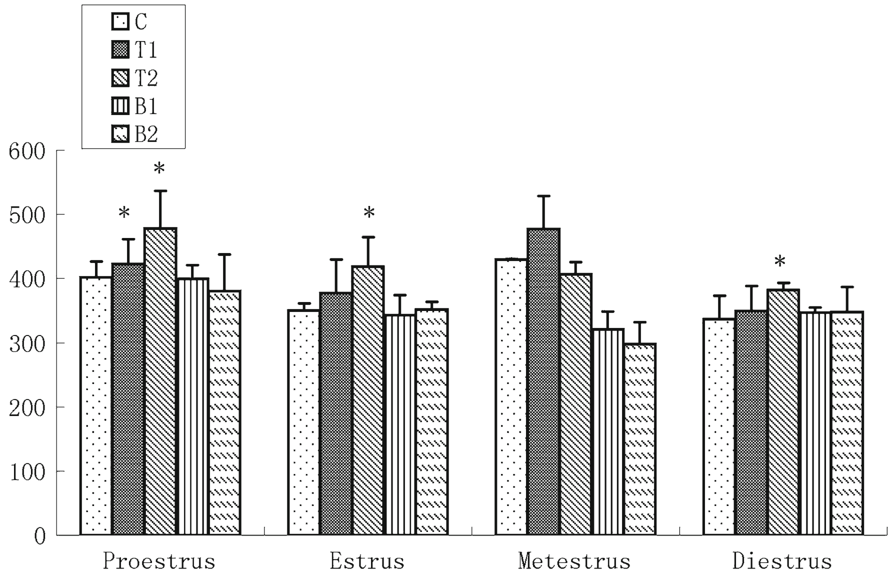

Fig. 2 Effects of taurine on the secretion of PRL in serum. Results are presented as mean ± SE (n = 6). *Significantly different from control group ($p < 0.05$)

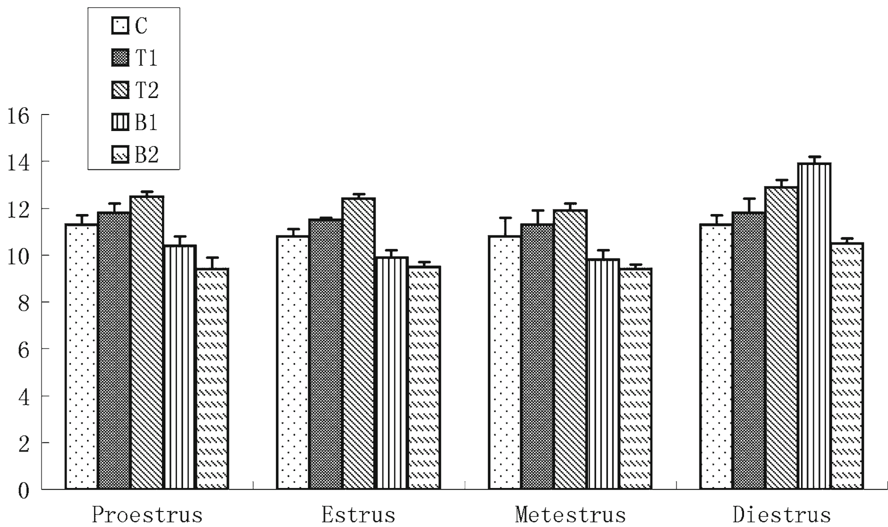

Fig. 3 Effects of taurine on the secretion of FSH in serum. Results are presented as mean ± SE (n = 6). *Significantly different from control group ($p < 0.05$)

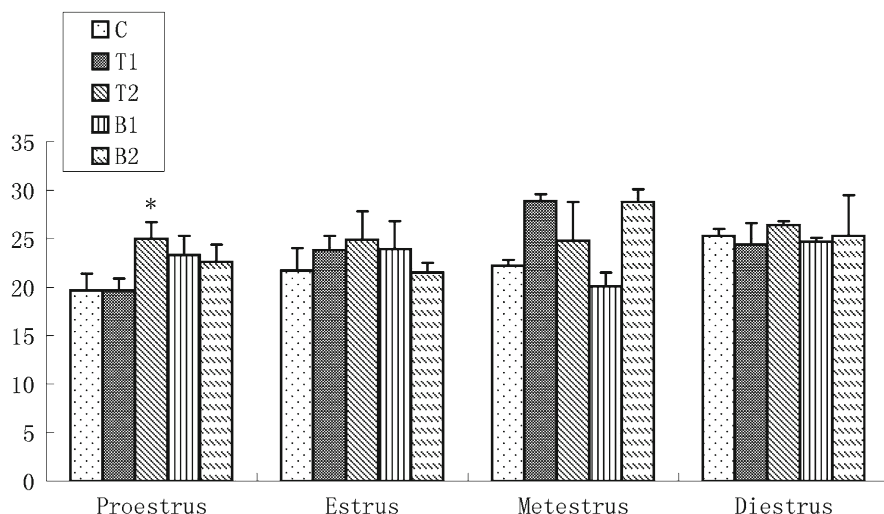

Fig. 4 Effects of taurine on the secretion of LH in serum. Results are presented as mean ± SE (n = 6). *Significantly different from control group ($p<0.05$)

As shown in Fig. 4, in Proestrus, the level of LH in the serum was significantly increased by taurine (Group T2) administration ($p<0.05$); but in Estrus, Metestrus and Diestrus, the level of LH showed no significant changes ($p>0.05$).

3.3 Results of Hormone Contents in Serum Secreted by the Ovaries

As shown in Fig. 5, in Proestrus and Estrus, the level of E2 in the serum was significantly increased by taurine (Group T2) administration ($p<0.05$); the Group T1 was significantly higher than Group C in Proestrus ($p<0.05$); but in Metestrus and Diestrus, the level of E2 showed no significant changes ($p>0.05$).

As shown in Fig. 6, in Metestrus and Diestrus Proestrus, the level of LH in the serum was significantly increased by taurine (Group T2) administration ($p<0.05$); but in Estrus and Proestrus, the level of LH showed no significant changes ($p>0.05$).

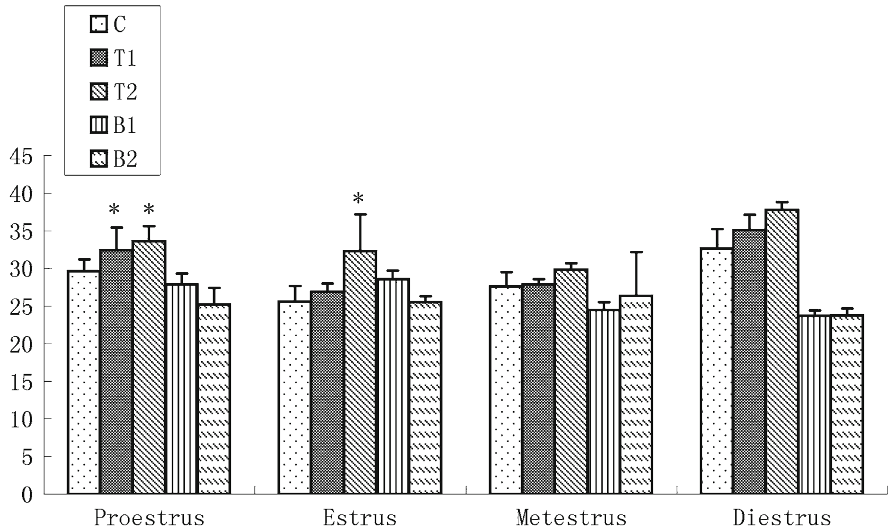

Fig. 5 Effects of taurine on the secretion of E2 in serum. Results are presented as mean ± SE (n = 6). *Significantly different from control group ($p < 0.05$)

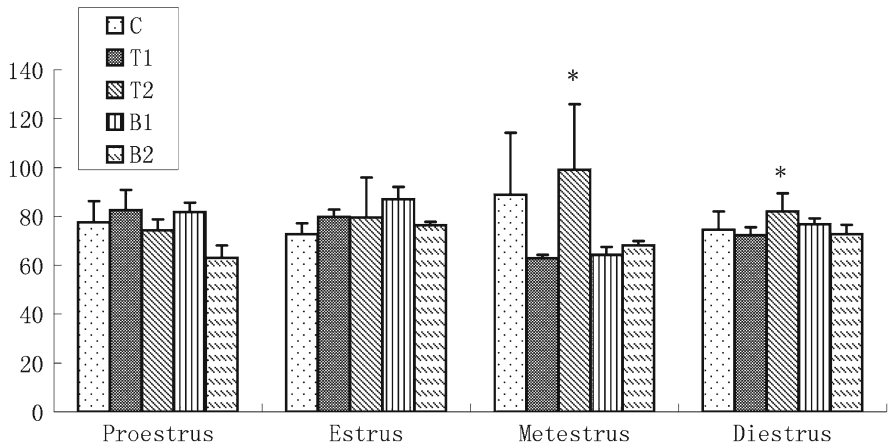

Fig. 6 Effects of taurine on the secretion of P in serum. Results are presented as mean ± SE (n = 6). *Significantly different from control group ($p < 0.05$)

4 Discussion

Taurine increases, while β-alanine decreases serum levels of GnRH in all four estrous stages in the female rat. GnRH is a neurohormone secreted by the hypothalamus. It promotes superovulation in females, estrus synchronization, and regulate FSH and LH selectively. It is speculated that taurine plays an important role on reproductive regulation of female animal.

The gonadal hormone of females that is secreted by adenohypophysis mainly include PRL, FSH and LH. From the PRL results (Fig. 2), it could be shown that taurine stimulated the level of secretion, while β-alanine inhibited the secretion level of PRL, FSH and LH. It has been reported that 15 min post injection of 5 μmol taurine into paracele of rats caused an increase in serum prolactin levels in male rats (Makinen et al. 1993). Injection of 0.25 and 1 μmol taurine into paracele of adult male rats stimulated the secretion of growth hormone and prolactin. However, when the injection rate exceeded 4 μmol, it inhibited GH and PRL secretion (Ikuyama et al. 1988). These data are consistent with our findings. Injected taurine into ventricle and anterior pituitary of female rat directly had no effect on the secretion of FSH and LH (Scheibel et al. 1980). In the discrepancies between these reports and our data could be explained by the differences in the uptake mechanisms and intestinal absorption.

For female animals, the role of PRL is mainly causing lactation, promoting generation of corpus luteum and maintaining the secretion of progestational hormone; FSH is to stimulate growth and maturation of follicle and to promote ovulation; LH is to promote the secretion of estrogen. The experimental results show that taurine can promote secretion of PRL, FSH and LH in female rats. The promotion effect is more significant especially in T2 group. Therefore, it can be speculated that taurine could have a direct effect on the pituitary, and contribute to reproductive regulation in female rats.

E2 is the most important estrogen secreted by the ovaries. P is progestational hormone of the highest biological activity which is secreted by ovaries. The effect of both hormones is influenced by the synergistic effect of FSH and LH. The results showed that taurine could stimulate the level of E2 and P, while β-alanine reduced the level of E2 and P.

It has been reported that taurine could promote hen's development of ovarian tissue morphology and the secretion of estrogen (Xiao et al. 1997). The animals in the experiment were different, but the results and the test results were consistent. The results indicate that taurine may act on ovaries directly on one hand, promoting ovarian development; on the other hand, because taurine stimulates the pituitary to secrete LH and FSH, LH promotes the synthesis of follicular theca and secretion of testosterone, FSH activates aromatase in the granulosa cells, testosterone gets into the granulosa cells and converts to E2 under the action of aromatase.

5 Conclusion

Effects of taurine on female reproduction may be achieved mainly by regulating the activities of hypothalamic-pituitary-ovarian axis-related hormones, and its exact mechanisms need to be further investigated.

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Taurine Enhances Proliferation and Promotes Neuronal Specification of Murine and Human Neural Stem/Progenitor Cells

Herminia Pasantes-Morales, Gerardo Ramos-Mandujano, and Reyna Hernández-Benítez

Abbreviations

BrdU	Bromodeoxyuridine
bFGF	Basic fibroblast growth factor
CFSE	Carboxyfluorescein diacetate succinimidyl ester
DAVID	Database for annotation, visualization and integrated discovery
EGF	Epidermal growth factor
GFAP	Glial fibrillary acidic protein
JC1	5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimi-dazolylcarbocyanine iodide
KEGG	Kyoto encyclopedia of genes and genomes
LeX	Lewis X
MAP2	Microtubule-associated protein 2
MBP	Myelin basic protein
mtRNA	Mitochondrial transfer Ribonucleic acid
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide
NAO	10-N-nonyl acridine orange
NPCs	Neural precursor cells
Pax6	Paired box protein 6
Shh	Sonic Hedgehog
SVZ	Subventricular zone
TAUT	Taurine transporter
UBP	Ubiquitin/proteosome
Wnt	Wingles-Integrase1

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1 Introduction

A number of taurine functions in animal tissues have been so far described, which include: (1) Taurocholic acid production, (2) Cell volume regulation, (3) Modulation of cation transport, (4) Cellular membrane stabilization, (5) Reduction of lipid peroxidation and oxidative stress, (6) Regulation of the immune system, (7) Increasing mitochondrial efficiency, (8) Neuromodulation.

2 Taurine Implications in Brain Development

Taurine was first associated with brain development by studies showing the higher taurine content (3–5-times higher) in the developing and neonatal brain as compared to the adult brain. This was a pattern consistently observed in spite of the large differences in taurine levels found between species (Agrawal et al. 1968; Sturman and Gaull 1975; Miller et al. 2000). Other tissues do not have this kind of developmental change in the taurine concentration. These observations pointed to a requirement for taurine when brain cells are in the process of development or maturation, a notion which was later conclusively demonstrated by a series of studies showing impaired brain development in taurine deficient animals (Table 1). Although the taurine requirement for optimal brain development is well established, the mechanisms affected by taurine deficiency remain unclear. Taurine actions as a trophic factor and/or a protective factor might be involved in the taurine requirement for optimal maturation of neural cells (Pasantes-Morales and Hernandez-Benitez 2010). This question has been addressed in our and others recent studies exploring taurine effects in stem and progenitor cells of neural origin. These cells reproduce the proliferation and maturation processes occurring in the developing brain. Studies have been conducted in neural stem/progenitor cells, altogether named as neural precursor cells (NPCs), obtained from the embryonic mouse mesencephalon (Hernandez-Benitez et al. 2010b), the hippocampus dentate gyrus (Shivaraj et al. 2012) and the human fetal brain (Hernandez-Benitez et al. 2013). Also taurine effects had been observed in NPCs from the SVZ of the lateral ventricles of adult brain, region in which neurogenesis persist until adult stage (Hernandez-Benitez et al. 2012).

The present review summarizes the results of these studies regarding to the taurine effect on cell viability, proliferation, lineage potential, and possible mechanisms of taurine on NPCs.

3 Generation of NPCs from Different Brain Tissues and Their Taurine Content

NPCs can be isolated from different regions of the developing brain or from specific areas of mature brain (Ahmed 2009). To obtain NPCs cultures, the excised tissue is maintained in a serum-free medium, in the presence of the growth factors EGF and

Table 1 Taurine in implications in brain and central nervous system

Species	Condition	Affected tissue/cells	Consequence	References
Cat	2 weeks old kittens from taurine-deficient mothers	Cerebellum and brain cortex	Delay in cell cycle. Deficient migration	Smith and Downs (1978)
	8 weeks old kittens from taurine-deprived mothers	Cerebellum	Inadequate cell migration and organization	Sturman et al. (1985)
		Spinal cord	Disturbed morphology and abnormal alignment of the dorsal root nerves	
		Visual cortex	Delayed migration, organization and differentiation	Palackal et al. (1986)
			Low number of pyramidal cells and poor arborisation	Palackal et al. (1985)
	Taurine deficient fetuses	Brain	Severe hydrocephaly and cases of anencephaly	Sturman et al. (1987)
Monkeys	Dietary deprived of taurine	Visual cortex	Defective organization of cortical layers	Neuringer et al. (1990)
Rat	Antenatal taurine supplementation in fetuses with intrauterine growth restriction	Brain	Improving IUGR fetal brain development	Liu et al. (2011).
Cat	Fed synthetic taurine free diets	Retina	Degeneration	Hayes et al. (1975)
	Feeding with dog food		Generalized retinal atrophy. Blindness	Aguirre (1978); Ricketts (1983)
	Taurine-depleted		Membrane disorganization of tapetal rods	Wen et al. (1979); Sturman et al. (1981)
Monkey	Fed a protein-deficient semi-purified diet		Defective electroretinogram	Neuringer et al. (1979)
	Raised from birth on a synthetic taurine-free milk formula		Degeneration of the retinal cone photoreceptor cells	Sturman et al. (1984)
Rat	Weanling rats fed a low protein diet		Depressed α and β waves in the electroretinogram	Bankson and Russell (1988)
	Degeneration of retinal ganglion cells in culture or in vivo		Improved survival of retinal ganglion cells	Froger et al. (2012)
Human	Fed with taurine-free milk formula		Ophthalmologically and electrophysiologically demonstrable retinal damage	Geggel et al. (1982)

(continued)

Table 1 (continued)

Species	Condition	Affected tissue/cells	Consequence	References
Rat	Antenatal taurine supplementation in fetuses with intrauterine growth restriction	Neurons	Improved neuronal regeneration	Liu et al., (2014)
Mouse	Primary cultures	Cerebellar cells	Defective migration	Maar et al. (1995)
Human		Neurons	Increasing or restoring cell proliferation	Chen et al. (1998)

bFGF, which sustain the cell proliferation of neural precursors (Rietze and Reynolds 2006). Under these conditions, most cells from the dispersed tissue are not responsive to these factors and die. Only a small amount of cells (typically less than 1 %) survive and proliferate, forming spherical free-floating aggregates known as neurospheres. Each neurosphere is formed by a variable proportion of stem cells and progenitor cells, which as above mentioned are together named NPCs. Cells in neurospheres are typically characterized by markers such as nestin, CD133, Sox2, Musashi, LeX, and Pax6 (Ramasamy et al. 2013). NPCs in neurospheres are able to proliferate, self-renew, and further differentiate into astroglia, oligodendroglia and neurons.

In our studies, the cultured neurospheres were derived from cells obtained from the embryonic mouse mesencephalon, the subventricular zone (SVZ) of adult mouse brain, and the human fetal brain (Hernandez-Benitez et al. 2010b, 2012, 2013). In those cultures, neurosphere-forming cells were positive to nestin in a proportion of over 94 % in the embryonic model, 93 % in the adult model, and 76 % in the fetal human model. On this basis, cells were considered mostly as NPCs.

Taurine content in NPCs and in the tissue from which those cells are derived is compared in Fig. 1. Cells from the freshly dissected mouse mesencephalon (13.5 embryonic days) contained high taurine levels as is characteristic of the developing brain. In cells from the adult brain, obtained from the subventricular zone, the main region where NPCs reside in adults, taurine level are notably lower. These data are in agreement with previous studies on murine brain taurine concentration (Agrawal et al. 1968; Miller et al. 2000). The taurine concentration quantified in three human fetal samples (Hernandez-Benitez et al. 2013) showed a taurine content of 5 mM, a number close from the 6 mM reported by Sturman and Gaull (1975) for the fetal human brain at the second trimester.

After a few days in culture, neurospheres derived from all the three different tissues contain very low taurine levels, evidencing that the typical neurosphere system is a taurine deficient-model. Addition of taurine to embryonic and fetal cultures invariably results in the recovery of the taurine content typical of brain cells during

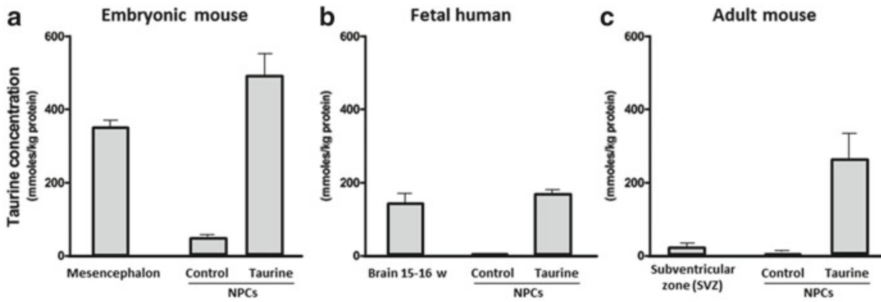

Fig. 1 Taurine levels in NPCs and in brain source tissue. Taurine levels were measured by HPLC in tissue samples or neurospheres obtained from (**a**) mesencephalon of embryonic mouse (E13.5 day), (**b**) fetal human brain of 15–16 weeks, and (**c**) SVZ of the lateral ventricles of adult mouse brain. Tissue samples or cultured neurospheres were collected, washed, and sonicated. The protein content in each sample was eliminated by alcoholic precipitation. Then, each sample was derivatized with o-phthaldialdehyde (1:1), and injected into a Beckman liquid chromatograph system, using the following specifications: ODS column of 4.6 × 250 mm internal diameter; fluorescence detector with emission at 460 nm and excitation at 330 nm; mobile phase of methanol/potassium acetate (0.1 M, pH 5.5); running rate of 1.5 mL/min with a linear gradient for 15 min. Protein content was determined by the Bradford procedure

development (Fig. 1). This capacity to accumulate taurine indicates the presence of an active mechanism of taurine uptake, likely by the energy-dependent taurine transporter TAUT (Tappaz 2004). This transporter was found expressed and functionally characterized in the embryonic and adult mouse NPCs (Hernandez-Benitez et al. 2010a, 2012).

4 Taurine Effects on Neurosphere Cultures

Cultures grown in the presence of taurine increase the number and size of neurospheres. Accordingly, the number of cells in cultures counted after neurosphere disaggregation was found consistently increased. This is observed in cultures derived from embryonic or adult cells, from murine or human origin, although the magnitude of the increase is different. All cultures start with a similar number of cells, and after 96 h, taurine cultures contained about 38, 104, and 120 % more cells than control cultures in the embryonic, fetal, and adult model, respectively (Fig. 2a). The presence of taurine in neurosphere cultures in the absence of growth factors does not induce per se the formation of neurospheres nor does it change the typical expression pattern of NPC markers.

The described effect of taurine increasing the number of cells in culture could be the consequence of an increase in proliferation and/or on cell viability. Examination of both parameters was carried out in the murine models (Table 2).

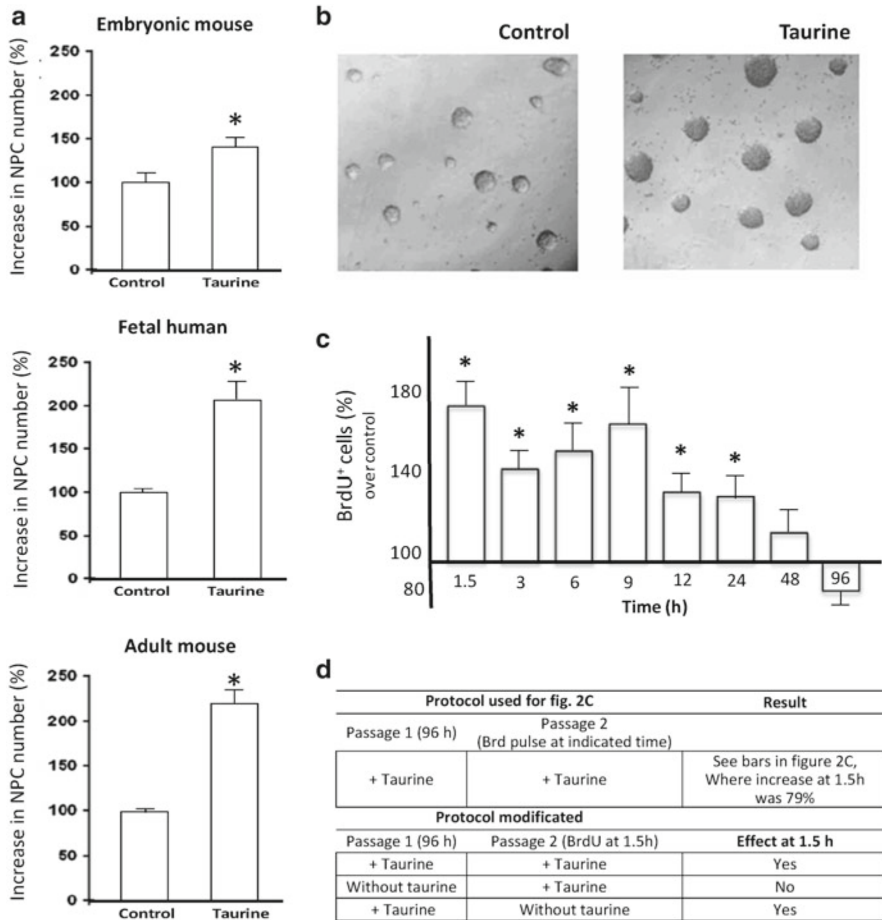

Fig. 2 Effect of taurine on NPC number in cultures. (**a**) *Bars* represent the percentage increase in NPC number in cultures grown in the presence of 10 mM taurine over the control condition. (**b**) Representative images of neurosphere cultures in control and taurine conditions. (**c**) Time course of cellular BrdU incorporation. Results are expressed as percentage of $BrdU^+$ cells in taurine condition over control cultures. A single pulse of BrdU was applied 1.5 h before the end of the experiment at the indicated times, at which point the $BrdU^+$ cells were evaluated. (**d**) Protocols used regarding the time of taurine exposure in culture and evaluated as in (**c**), but only at the time of maximal taurine effect (1.5 h). *Bars* represent the mean values ± SEM of 3–6 separate experiments. Differences between the conditions according to a Student t-test (*$p < 0.05$)

The viability of NPCs was determined by various experimental assays, including direct counting (trypan blue exclusion assay), spectrophotometric measurements (MTT), and flow cytometry determinations (propidium iodide, calcein-AM, annexin-V). Only the results derived from flow cytometry on NPCs from the adult brain showed a small effect of taurine increasing survival and decreasing apoptosis (Table 2). However, this effect of taurine on viability could not entirely explain the notably high number of cells in taurine cultures.

Table 2 Taurine effects on proliferation and survival of NPCs

Parameter	Reagent	Method	Origin of NPCs	Result
Proliferation	BrdU	Immunocytochemistry	Embryonic mouse Adult mouse	DNA synthesis increase 37 % DNA synthesis increase 79 %
Cell cycle analysis	Propidium iodide	Flow cytometry	Adult mouse	S-phase increase 46 % G2/M-phase increase 30 % G0/G1-phase decrease 8 %
	CFSE			No effect of taurine on the cell cycle duration
Necrosis	Trypan blue	Direct counting	Embryonic mouse Adult mouse Fetal human	No effect
	Propidium iodide	Flow cytometry	Adult mouse	Necrotic cells decrease 20 %
Apoptosis	Propodium iodide	Flow cytometry	Fetal human	No effect
	Annexin-V FITC	Flow cytometry	Adult mouse	Apoptotic cells decrease 40 %
Viability	MTT	Colorimetric assay	Adult mouse	No effect
	Calcein-AM	Flow cytometry	Adult mouse	Viable cells increase 15 %

The proliferation rate of NPCs derived from adult mice brain was markedly increased by taurine. Flow cytometry analysis of the DNA content showed that taurine increased the proportion of cells in the S-phase and reduced the proportion of cells in the G0/G1 phase, without affecting the cell cycle duration (Table 2). This effect of taurine increasing proliferation is also evident by the BrdU assay. A time course of BrdU incorporation in NPCs provided interesting information about the effect of taurine in cell proliferation. BrdU incorporation is higher in taurine-containing cultures during essentially all the time in culture, but the effect is markedly higher during the first hours, being maximal after only 1.5 h after seeding (Ramos-Mandujano et al. 2014). NPCs in the absence of taurine also proliferate but with a delay of more than 12 h with respect to taurine cultures. These results suggest that there is no absolute requirement of taurine for cell proliferation but that cells grown in the presence of taurine appear to be better suited for progressing into the normal phases of the proliferation cell cycle. Cultures in these experiments grow in the presence of taurine all the time after seeding and up to the 96 h of duration of the experiment. The presence of taurine only during the first 1.5 h in culture is not sufficient to sustain its effect increasing BrdU incorporation. This observation suggests that the action of taurine requires a prolonged interaction with cells and is not the result of short-term effects such as those that could result from membrane stabilization or from activation of membrane-located proliferation receptors. Therefore, other possibilities had to be explored.

5 Taurine Effect on the Lineage Proportions of Differentiated Cells

The ability to generate the different cell types of central nervous system is a distinctive characteristic of NPCs (Reynolds et al. 1992). In both NPCs from mouse and human, the differentiation was induced by replacing the proliferation medium by a medium without growth factors and containing 1 % bovine serum (Hernandez-Benitez et al. 2012, 2013). After 7–8 days in culture, the number of differentiated cells was evaluated by immunocytochemistry, by counting the number of cells positive for markers such as GFAP for astrocytes, βIII-tubulin or MAP2 for neurons, and MBP for oligodendrocytes. Typically, after a differentiation process, astrocytes are the most abundant cell type, while fewer neurons are present. However, when the differentiation medium is supplemented with taurine, the proportion of neurons formed has significantly increased in the fetal and adult models (Table 3).

6 Mechanisms of the Taurine Action on NPCs: the Mitochondria as Main Target

The above described studies appear to discard the effects of taurine as membrane stabilizer. Another possibility discarded is that of an effect of taurine at the nucleus via a direct interaction with nuclear elements. This is a conclusion raised by the demonstration that taurine is located at the cytosol and absent from nuclei

Table 3 Taurine increases neurogenesis in NPCs cultured in differentiation medium

NPC model	Protocol	βIII tubulin^{+} cells % in control	Increase over control induced by taurine (10 mM)	Reference
Embryonic mesencephalon (mouse)	Neurospheres were generated in PM ± taurine, and then NPCs were plated in DM for 7 days	10–12	No increase	Hernandez-Benitez et al. 2010b
Fetal (human)	NPCs were plated in DM ± taurine for 8 days	4.8	307 %	Hernandez-Benitez et al. 2013
ReNcell (human cell line)	ReNcells were plated in DM ± taurine for 4 days	7.4	123 %	Hernandez-Benitez et al. 2013
Adult SVZ (mouse)	NPCs were plated in DM ± taurine for 7–8 days	3.1	229 %	Hernandez-Benitez et al. 2012

PM proliferation medium, *DM* differentiation medium

(Ramos-Mandujano et al. 2014). An alternate approach to investigate the mechanisms of taurine action is the microarray gene expression analysis. This type of analysis has provided information about the effect of taurine on signaling pathways involved in cells from heart, liver, skeletal muscle and kidney, and in the cell lines HepG2 and Caco-2 (Park et al. 2006; Warskulat et al. 2006; Mortensen et al. 2010a, b; Gondo et al. 2012; Han and Chesney 2013; Liang et al. 2013).

The first microarray on neural cells exploring the role of taurine was developed in NPCs isolated from the subventricular zone of adult mice brain (Ramos-Mandujano et al. 2014). This analysis showed that taurine regulates NPC genes implicated in proliferation, survival, adhesion, and mitochondrial functioning (Table 4, and complete results at GEO, accession number GSE53547). Interestingly, if the group of genes with Z-score higher than 2 is submitted to Gene Ontology analysis, the KEGG database of DAVID retrieves a group of mitochondrial genes, which although being small, are those exhibiting the most significant enrichment. A previous microarray analysis correlated taurine with mitochondrial genes in cells from liver and skeletal muscle of newborn mice subjected to low protein maternal diet (Mortensen et al. 2010a, b). In that study, the newborn protein-restricted mice showed significant changes in mitochondrial genes, which are prevented by taurine. Also, some mitochondrial genes were found up regulated after gestational taurine supplementation, even in the control offspring (Mortensen et al. 2010b).

These results fit into the proposed hypothesis about an effect of taurine increasing the number of cells suitable for proliferation, via a better performance of mitochondria, resulting in the activation of some common proliferation pathways, such as Shh and Wnt (Table 4). This hypothesis was further corroborated measuring the fluorescence of Rhodamine123/NAO and JC1, as markers of the mitochondrial performance in NPCs. Results revealed an increase over 40 % in the cell population with a higher mitochondrial potential value in taurine NPCs as compared to controls (Ramos-Mandujano et al. 2014). Therefore, mitochondrial functioning may be the initial site affected by taurine, then leading to the activation of signaling pathways related to proliferation.

The involvement of taurine in mitochondrial function has been recently considered, based on the early study by Suzuki et al. (2002, 2011), showing the modification by taurine of the uracil ring at the anticodon wobble position of the mtRNAs for leucine, lysine, glutamate and glutamine. This action improves the production of mitochondrial proteins involved in the respiratory chain elements, making more efficient the electron-chain transport and ATP formation. This could represent an advantage for NPCs in the G1 phase of the cell cycle and promote the subsequent transition into later cell cycle phases, increasing the cell DNA synthesis and raising the number of NPCs in cultures.

Similarly, a more efficient mitochondrial functioning could be a decisive factor on NPCs subjected to differentiation, since neuronal survival in cultures is markedly affected by the energy state of the cell during the differentiation phase (Ostrakhovitch and Semenikhin 2012). The microarray analysis on taurine-containing NPCs showed down regulation of genes related to the ubiquitin/proteosome (UBP) system (Table 4), an essential regulator of apoptosis that plays a critical role in cell survival

Table 4 Genes modified by taurine on NPCs

Proliferation	Survival	Adhesion	Mitochondrial function
X76290 Sonic hedgehog	AK013153 death associated protein kinase 1	AK020282 receptor (calcitonin) activity modifying protein 1	AF133093 L1 cell adhesion molecule; isocitrate dehydrogenase 3 (NAD+), gamma
NM_007982 PTK2 protein tyrosine kinase 2	NM_007982 PTK2 protein tyrosine kinase 2	NM_007835 dynactin 1	BC014818 NADH dehydrogenase (ubiquinone) flavoprotein 1
AF133093 L1 cell adhesion molecule; isocitrate dehydrogenase 3 (NAD+)γ	AK018592 TNFRSF1A-associated via death domain	NM_007982	NM_007747 cytochrome c oxidase, subunit Va
AF002823 budding uninhibited by benzimidazoles 1 homolog	NM_009684 apoptotic peptidase activating factor 1	PTK2 protein tyrosine kinase 2	BC002163 NADH dehydrogenase (ubiquinone) Fe-S protein 5
NM_010568 insulin receptor	AK016520 cullin 2	AF133093 L1 cell adhesion molecule; isocitrate dehydrogenase 3 (NAD+), gamma	BC003423 ubiquinol cytochrome c reductase core protein 2
AF090738 insulin receptor substrate 2	AK011758 protein phosphatase 2, gamma isoform	AK003152 titin	NM_009743 BCL2-like 1
NM_015752 suppressor of fused homolog	AK011647 protein kinase, cAMP dependent regulatory, type II beta	X85992 sema domain, immunoglobulin domain (Ig), (semaphorin) 4B	NM_007822 cytochrome P450, family 4, subfamily a, polypeptide 14
NM_010296 GLI-Kruppel family member GLI1	NM_011101 protein kinase C, alpha	NM_016780 integrin beta 3	AK018195 dynamin 1-like
NM_023653 wingless-related MMTV integration site 2	NM_009743 BCL2-like 1	NM_013566 integrin beta 7	NM_007807 cytochrome b-245, beta polypeptide
AK011647 protein kinase, cAMP dependent regulatory, type II beta	NM_020272 phosphoinositide-3-kinase, catalytic, gamma polypeptide	NM_019767 actin related protein 2/3 complex, subunit 1A	NM_011169 prolactin receptor
NM_011101 protein kinase C, alpha	AK018164 phosphatidylinositol 3-kinase, catalytic, alpha polypeptide	D21796 ryanodine receptor 1, skeletal muscle	

(continued)

Table 4 (continued)

Proliferation	Survival	Adhesion	Mitochondrial function
AF056187 insulin-like growth factor I receptor	NM_007611 caspase 7	AK009080 sarcoglycan, beta (dystrophin-associated glycoprotein)	
NM_016891 protein phosphatase 2 (formerly 2A), regulatory subunit A	NM_008413 Janus kinase 2	NM_011655 tubulin, beta 5	
NM_009771 beta-transducin repeat containing protein	M81483 protein phosphatase 3, catalytic subunit, Bisoform	NM_021274 chemokine (C-X-C motif) ligand 10	
AF253540 adenylate cyclase 3	AK008417 forkhead box O3	AK013439 tubulin, beta 1	
NM_008413 Janus kinase 2	U38501 guanine nucleotide binding protein (G protein), alpha inhibiting 1	AF022804 phospholipase C, beta 4	
M81483 protein phosphatase 3, catalytic subunit, beta isoform	NM_010431 hypoxia inducible factor 1	NM_010718 LIM motif-containing protein kinase 2	
NM_011104 RIKEN cDNA 9630025F12 gene; protein kinase C, epsilon	NM_007829 Fas death domain-associated protein	NM_007911 ephrin B3	
NM_009525 wingless-related MMTV integration site 5B	NM_016786 ubiquitin-conjugating enzyme E2K	AK018195 dynamin 1-like	
NM_008058 frizzled homolog 8	AF288694 ubiquitin-activating enzyme E1-like	X95346 phospholipase C, gamma 1	
NM_007829 Fas death domain-associated protein	NM_009688 X-linked inhibitor of apoptosis	U38501 guanine nucleotide binding protein (G protein), alpha inhibiting 1	
X12616 feline sarcoma oncogene	AF244362 ubiquitin protein ligase E3B	NM_011339 chemokine (C-X-C motif) ligand 15	
M59912 kit ligand	AK020784 UL16 binding protein 1	NM_021895 actinin alpha 4	
NM_007790 predicted gene 8892; structural maintenance of chromosomes	NM_018812 similar to potassium channel regulatory protein KChAP; inhibitor of activated STAT 3	NM_009137 chemokine (C-C motif) ligand 22	

(continued)

Table 4 (continued)

Proliferation	Survival	Adhesion	Mitochondrial function
NM_007462 similar to adenomatosis polyposis coli	NM_009017 retinoic acid early transcript 1E; retinoic acid early transcript beta	X93167 fibronectin 1	
NM_009828 cyclin A2	AY039762 24-dehydrocholesterol reductase	AF141322 caveolin 2	
AF141322 caveolin 2	NM_009425 tumor necrosis factor ligand superfamilym10	NM_020293 claudin 9	
NM_009521 wingless-related MMTV integration site 3	AK011961 ubiquitin-conjugating enzyme E2B, RAD6 homology	NM_031163 collagen, type II, alpha 1	
AF015948 E2F transcription factor 3	NM_010762 myelin and lymphocyte protein, T-cell differentiation protein	AB051897 chemokine (C-C motif) ligand 5	
NM_011379 signal-induced proliferation associated gene 1	AK004018 tripartite motif-containing 37	NM_008816 platelet/endothelial cell adhesion molecule 1	
NM_008357 interleukin 15	NM_008840 phosphatidylinositol 3-kinase catalytic delta polypeptide	AF119148 filamin C, gamma	
NM_021505 anaphase-promoting complex subunit 5	NM_016679 kelch-like ECH-associated protein 1	X03766 actin, alpha 1, skeletal muscle	
NM_011799 cell division cycle 6 homolog	NM_011609 tumor necrosis factor receptor superfamily, member 1a	X57277 RAS-related C3 botulinum substrate 1	
NM_053116 wingless-related MMTV integration site 16	NM_026305 predicted gene 11401; similar to transcription elongation factor B (SIII)	AF255911 junction adhesion molecule 2	
NM_009067 ralA binding protein 1	NM_010937 similar to neuroblastoma ras oncogene; neuroblastoma ras oncogene	J04695 collagen, type IV, alpha 2	
X57277 RAS-related C3 botulinum substrate 1	NM_010786 transformed mouse 3T3 cell double minute 2	NM_015734 collagen, type V, alpha 1	
AK014910 speedy homolog A	AK014894 cullin 5	NM_007831 deleted in colorectal carcinoma	

(continued)

Table 4 (continued)

Proliferation	Survival	Adhesion	Mitochondrial function
AF175892 protein kinase, membrane associated tyrosine/threonine 1	AK004127 thyroid hormone receptor interactor 12	AF028129 similar to natural killer cell receptor Ly49C	
AK020732 insulin-like growth factor I receptor	Z11664 son of sevenless homolog 2	M31585 intercellular adhesion molecule 1	
AK008943 secreted frizzled-related protein 1	NM_025830 WW domain containing E3 ubiquitin protein ligase 2	NM_007615 catenin (cadherin associated protein),δ1	
NM_009403 tumor necrosis factor (ligand) superfamily, member 8		NM_011169 prolactin receptor	
NM_009861 cell division cycle 42 homolog ; predicted gene 7407		AF288377 killer cell lectin-like receptor subfamilyAm4	
NM_007615 catenin (cadherin associated protein), delta 1		AK009330 claudin 23	
NM_010786 transformed mouse 3T3 cell double minute 2		U88684 immunoglobulin heavy chain 3 (serum IgG2b)	
NM_010206 fibroblast growth factor receptor 1		NM_007614 catenin (cadherin associated protein)β1	
NM_008359 interleukin 17 receptor A			
X59047 CD81 antigen			
Z11664 son of sevenless homolog 2			
J03783 interleukin 6			
NM_010284 growth hormone receptor			
AF250844 mutL homolog 1			
NM_007614 catenin (cadherin associated protein), beta 1			

The expression pattern was determined by a microarray analysis and its original results are available on the database of Gene Expression Omnibus (GEO, accession number GSE53547). Taurine regulates around 2,200 genes, which were analyzed by Gene Ontology tools to find the possible pathways implicated in the taurine actions on NPCs. This analysis sorted 170 genes that enriched functional cellular pathways which were classified according their general function

in cultures (Canu et al. 2000; Vucic et al. 2011). This effect is consistent with the reduction in apoptosis and necrosis observed by flow cytometry determinations in taurine cultures (Ramos-Mandujano et al. 2014). Increasing cell viability may explain the higher number of neurons found in taurine cultures upon differentiation (Hernandez-Benitez et al. 2012, 2013).

7 Conclusions

Results from our studies here revised show that restoration of taurine levels in cultured NPCs up to the level found in fresh tissue, has a beneficial effect increasing the number of cells in cultures. A variety of experimental approaches, including gene expression analysis, led us to conclude that taurine is positively influencing the mitochondrial performance, improving the ability of cells to transit into the phases of proliferation cycle. Taurine also increased the number of neurons formed after differentiation of NPCs. This effect may also be a consequence of a more efficient mitochondrial operation, thus increasing neuronal viability, which is known to be highly dependent on a good energy supply.

Taurine effects here described may also occur in vivo in the immature brain and could explain in part its requirement for optimal brain development. The findings in our studies are also relevant to therapeutic strategies of neural replacement with neurons differentiated from NPCs, in which taurine could be considered as a factor to enrich the niches of neurogenesis.

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Effects of Chronic Taurine Administration on Gene Expression, Protein Translation and Phosphorylation in the Rat Hippocampus

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Abbreviations

Akt	Protein kinase B
BDNF	Brain-derived neurotrophic factor
CaMKII	Calcium/calmodulin-dependent protein kinase II

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CCL6	Chemokine (C-C motif) ligand 6
CD80	Cluster of differentiation 80
CMTM2a	Chemokine-like factor-like MAL and related proteins for vesicle trafficking and membrane link domain transmembrane domain containing 2a
CRABP1	Cellular retinoic acid-binding protein 1
CREB	cAMP-response element-binding protein
EAPP	E2F-associated phosphoprotein
ERK1/2	Extracellular signal-regulated kinase1/2
GSK3β	Glycogen synthase kinase3-beta
HDAC	Histone deacetylase
PKC	Protein kinase C
PLAC8	Placentanspecific 8
QOL	Quality of life
SPP1	Secreted phosphoprotein 1
SSRI	Selective serotonin reuptake inhibitor

1 Introduction

Depressive disorder is a serious psychiatric disease and about 20 % of the victims suffers from depression (Berton and Nestler 2006). Antidepressants developed by pharmaceutical companies have been widely used especially in advanced countries. Unfortunately, some antidepressants including SSRIs have severe side effects such as nausea and anorexia (Vaswani et al. 2003), thus development of drugs without side effects is necessary for keeping QOL of patients. Also, life style is a key point for prevention of mental disorders; therefore diet, exercise, and sleep are thought to be important factors for mental condition. Especially, the values of nutrients and foods should be discovered, because nutrients and foods consisting of antidepressant-like activity have not yet been well studied.

We developed some animal models of depression using resident-intruder paradigm and characterized them (Iio et al. 2011, 2012a, 2014a b; Goto et al. 2014). Using metabolomics analyzes, we discovered some changes of liver metabolism in the depression model of rodents (Iio et al. unpublished data). Especially, depression model of rat had elevated taurine concentration in the liver. Moreover, depression model of mice showed elevated taurocyamine in the liver (Goto et al. in press). Taurine is one of the most abundant free amino acids in the central nervous system (Hussy et al. 2000). Because taurine transporter is localized in the blood-brain barrier (Tamai et al. 1995), possibly orally administrated taurine can enter into the brain tissues. Also, taurine is synthesized from cysteine, and has various physiological functions such as membrane stabilization, osmoregulation and neuroprotection (Hussy et al. 2000; Timbrell et al. 1995; Tanabe et al. 2010). Moreover, taurine acts as a neurotransmitter agonist for glycine and gamma-aminobutyric acid receptors (Albrecht and Schousboe 2005; del Olmo et al. 2000). Additionally, taurine influ-

ences intracellular calcium movement and calcium-dependent signaling molecules in the brain (Wu and Prentice 2010). Therefore, we have studied the relationship between taurine metabolism and depression. In our previous studies, we elucidated that chronic taurine supplementation in rats has an antidepressive-like effects with some molecular changes of hippocampal signal transduction (Iio et al. 2012b). Taurine supplementation for 4 weeks (45 mmol/kg diet) increased phosphorylation of ERK1/2, Akt, GSK3β, CREB, and CaMKII in the hippocampus following reduced immobility in the forced swimming test (Iio et al. 2012b). Depressive disorders are induced by several biochemical changes such as neurotransmitters and neurotrophic factors. BDNF, one of the major neurotrophic factors in the brain is implicated in depression, and BDNF expression is decreased by exposure to various psychological stresses (Krishnan and Nestler 2008). On the other hand, injection of BDNF into the rodent hippocampus showed antidepressant-like effects (Shirayama et al. 2002). However, previous report describes that hippocampal BDNF expression is not changed (Murakami and Furuse 2010) and possibly it was downregulated by chronic taurine supplementation (Iio et al. unpublished data).

In this study, we tried to screen comprehensively the expression changes of genes and proteins in the hippocampus after chronic supplementation of taurine in rats using commercial antibody and DNA microarrays.

2 Materials and Methods

2.1 Animals

Five-week-old male Wistar rats were obtained from Charles River (Yokohama, Japan) and housed individually at room temperature (22 ± 2 °C), with lights on from 6:00 to 18:00 with *ad libitum* access to food and distilled water. For monitoring health condition of rats, body weight and food intake were measured at the end of the acclimation phase and at weekly intervals during taurine administration (data not shown). All experimental procedures followed the Guidelines of the Animal Care and Use Committee of Ibaraki University.

2.2 Experimental Design and Drugs

After 1 week of acclimation in our animal facility, animals were divided into two groups: Control group ($n=6$), and Taurine-fed group ($n=6$). The control group was fed a commercial diet (MF for antibody array or AIN-93G for DNA array; Oriental Yeast, Tokyo, Japan) for 4 weeks. The Taurine-fed group was fed a taurine-containing diet (45.0 mmol taurine/kg diet for antibody array or 3 % for DNA array) for 4 weeks. Taurine-contained pellet diets were processed and produced by the feed company Oriental Yeast (Tokyo, Japan).

2.3 Protein and RNA Preparation for Antibody and DNA Microarrays

After 4 weeks of taurine supplementation, all animals were subjected to molecular and biochemical analyzes for the microarrays. After the cervical dislocation, the rat whole brains were rapidly collected and chilled on ice and the hippocampi were dissected out. For comprehensive analyses of protein and gene expression, we used the commercial antibody microarray (Kinex™, Kinexus, Canada) and DNA microarray (Agilent Expression Array, Agilent, USA), respectively. Hippocampal tissues from three rats of each group were mixed in one plastic tube and pretreated as the instruction manuals for these microarrays (Kinexus and Agilent). Array data were produced and analyzed by Kinexus and Agilent.

3 Results and Discussion

3.1 The Effects of Chronic Taurine Supplementation on Expression and Phosphorylation of Hippocampal Proteins

Chronic supplementation of taurine affected some protein expression and phosphorylation in the hippocampus (Table 1). In this preliminary study, we analyzed the array data from mixed samples of each group, therefore we cannot determine statistical significance of difference between the control group and the taurine-fed group. Under this limitation, p53 and Jun were phosphorylated by chronic taurine. Especially, p53 was phosphorylated at Serine6 and Serine27 by taurine. p53 is a famous tumor suppressor protein and plays a crucial role in the regulation of cell cycle in multicellular organisms. The functional interaction of taurine and p53 in the hippocampus is unclear, although hippocampal p53 induces apoptosis of pyramidal neuron (Jordán et al. 1997). Previous report described that taurine transporter is a downstream target gene of p53 that is related to renal development and apoptosis (Han et al. 2002). Therefore, taurine may influence neuronal survival and repair of DNA damage through p53 in the hippocampus.

Also, hippocampal HDAC5 was phosphorylated at Serine498 by chronic taurine administration. HDAC5-dependent acetylation is related to the expression of BDNF gene in the hippocampus (Tsankova et al. 2006). Using chronic social defeated model of mice, hippocampal BDNF gene expression was reduced by chronic defeat following histone hypermethylation at the promoter region of BDNF gene (Tsankova et al. 2006). Imipramine, one of the tricyclic antidepressant drugs, selectively downregulated HDAC5 and reversed the expression of BDNF following hyperacetylation of histones (Tsankova et al. 2006). Moreover, phosphorylation of Serine259 and Serine498 in HDAC5 induces translocation of HDAC5 from nucleus to cytosol and

Table 1 Taurine affects the phosphorylation and expression of hippocampal proteins

Target protein name	Phospho site (human)	Full target protein name	Z-ratio (taurine_45, control)	Refseq	Uniprot link
p53	S6	Tumor suppressor protein p53 (antigenNY-CO-13)	2.26	NP_000537	P04637
Jun	S243	Jun proto-oncogene-encoded AP1 transcription factor	2.17	NP_002219	P05412
NFκB p65	S529	NF-kappa-B p65 nuclear transcription factor	2.03	NP_003989	Q04206
PKCθ	S676	Protein-serine kinase C theta	1.66	NP_006248	Q04759
PKCμ (PKD)	Pan-specific	Protein-serine kinase C mu (protein kinase D)	1.44	NP_002733	Q15139
p53	S37	Tumor suppressor protein p53 (antigenNY-CO-13)	1.25	NP_000537	P04637
Jun	Pan-specific	Jun proto-oncogene-encoded AP1 transcription factor	1.23	NP_002219	P05412
HDAC5	S498	Histone deacetylase 5	1.08	NP_001015053.1	Q9UQL6
Rb	S795	Retinoblastoma-associated protein 1	1.07	NP_000312	P06400
JNK1/2/3	Pan-specific	Jun N-terminus protein-serine kinase (stress-activated protein kinase (SAPK)) 1/2/3	1.06	NP_002741	P45983
Hsp60	Pan-specific	Heat shock 60 kDa protein 1 (chaperonin, CPN60)	−1.01	NP_002147	P10809
Hsp70	Pan-specific	Heat shock 70 kDa protein 1	−1.02	NP_005336	P08107
Hsp60	Pan-specific	Heat shock 60 kDa protein 1 (chaperonin, CPN60)	−1.03	NP_002147	P10809
STI1	Pan-specific	Stress induced phosphoprotein 1 (Hsc70/Hsp90 organizing protein (Hop)	−1.10	NP_006810	P31948

(continued)

Table 1 (continued)

Target protein name	Phospho site (human)	Full target protein name	Z-ratio (taurine_45, control)	Refseq	Uniprot link
CDK1 (CDC2)	Pan-specific	Cyclin-dependent protein-serine kinase 1	−1.22	NP_001777	P06493
Csk	Pan-specific	C-terminus of Src tyrosine kinase	−1.26	NP_004374	P41240
Hsp90α/β	Pan-specific	Heat shock 90 kDa protein alpha/beta	−1.28	NP_005339	P07900
PKCα	Pan-specific	Protein-serine kinase C alpha	−1.50	NP_002728	P17252
PDI	Pan-specific	Protein disulfide-isomerase	−1.53	NP_000909.2	P07237
Caveolin 2	Pan-specific	Caveolin 2	−1.55	NP_001224	P51636
Hsp90β	Pan-specific	Heat shock 90 kDa protein beta	−1.70	NP_031381	P08238
CDK1 (CDC2)	Pan-specific	Cyclin-dependent protein-serine kinase 1	−1.72	NP_001777	P06493
Catenin β1	Pan-specific	Catenin (cadherin-associated protein) beta 1	−2.06	NP_001895	P35222

transcription of HDAC5 target genes with increased histone acetylation in the nucleus accumbens (Renthal et al. 2007). Therefore, phosphorylation of HDAC5 by taurine may play a significant role in the antidepressive-like action of taurine, although taurine supplementation did not affect the expression of BDNF in the hippocampus derived from normal healthy mice (Murakami and Furuse 2010). In future, the functional interaction between taurine and BDNF in the stress model of animals including chronic social defeat model should be elucidated.

On the other hand, the expression of heat shock proteins and β-catenin was down-regulated by taurine supplementation (Table 1). Both catenin and cadherin are expressed and localized at synaptic junctions in the central nervous system (Murase et al. 2002). These proteins play an important role in the synaptic structures. Together with increased phosphorylation of proteins related to synaptic plasticity such as CaMKII and CREB by taurine (Iio et al. 2012b), chronic taurine may rearrange and modify synaptic junctions in the hippocampus, thus taurine may impact synaptic plasticity and hippocampal-dependent memory acquisition.

3.2 The Effects of Chronic Taurine Supplementation on Expression and Phosphorylation of Hippocampal Proteins

Using DNA microarray technique, chronic taurine supplementation affected some genes expression in the hippocampus (data not shown). As described above, we also observed the DNA microarray data from mixed samples of each group; therefore we cannot evaluate statistical significance from these preliminary data. Under this limitation, we selected the genes showing different expression level between the control and the taurine-fed group at log 2 ratio > 2 and gSigEval > 2 (Agilent). Some genes encoding EAPP, CMTM2a, PLAC8, and CCL6 were up-regulated by chronic taurine, while genes encoding PLAC9, CRABP1, CD80, and SPP1 were down-regulated. The functional interaction between these genes and taurine supplementation is largely unknown, thus the future studies should be focused on the taurine function related to transcriptional regulation of these genes in the hippocampus.

4 Conclusion

We found that chronic taurine supplementation at 45 mmol taurine/kg diet or 3 % for 4 weeks induces some changes of protein and gene expression in rat hippocampus (Table 1). Especially, the beneficial effects of chronic taurine supplementation in rats might be mediated by phosphorylation of p53, Jun, HDAC5, and so on in the hippocampus. Also, expression of many genes was changed by chronic taurine, thus it is necessary to elucidate the functions of these genes in the hippocampus under taurine supplemented condition.

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Potential Anti-aging Role of Taurine via Proper Protein Folding: A Study from Taurine Transporter Knockout Mouse

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Abbreviations

TauTKO	Taurine transporter knockout
WT	Wild type

1 Introduction

Taurine is the most abundant free amino acid in mammalian tissues. Body taurine is maintained by biosynthesis from methionine or cysteine in liver or some other tissues. Diet ingredients are also important to maintain the abundance taurine. Taurine has many biological actions such as, osmoregulation, calcium handling, neurotransmission, conjugation to bile acids, fatty acids, and anti-apoptosis (Huxtable 1992; Schaffer et al. 2000; Sjovall 1959). Previous studies demonstrated that taurine is beneficial against a variety of aging-related diseases, such as chronic heart failure, diabetes, atherosclerosis, etc. (Ito et al. 2012, 2014b; Murakami 2014). Meanwhile, taurine deficiency in cat that has no capacity of taurine synthesis led to some disorders such as cardiomyopathy (Pion et al. 1987). There are few studies that directly demonstrate the effect of taurine on aging (El Idrissi et al. 2009, 2013), while the anti-aging effects of taurine have been proposed from these observations.

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We investigated the influence of tissue taurine depletion on tissue aging by using TauTKO mice. In this chapter, we will introduce the impacts of tissue taurine depletion on aging and lifespan in mice.

Furthermore, recent studies indicate that impairment of protein homeostasis contributes to tissue aging and some aging-related diseases (Lopez-Otin et al. 2013). Organic osmolytes, including taurine, not only regulate the balance of intracellular inorganic ions but also contribute to stabilization of macromolecules, including proteins (Burg and Ferraris 2008; Yancey 2005). Therefore, this action may be a key role in the anti-aging effects of taurine. In the latter part of the chapter, we will discuss the chaperonic effect of taurine and the impact of taurine deficiency on protein folding.

2 Aging Phenotype of Taurine Transporter Knockout Mouse

2.1 Lifespan

To clarify the role of taurine in aging, we investigated whether tissue taurine depletion affects the lifespan and tissue senescence by using TauTKO mice (Ito et al. 2014a). We first monitored the life span of TauTKO mice and control littermates. Survival data for males showed that the median life-span in TauTKO mice decreased by 154 days (from 683 to 529 days) relative to that of control litermates (WT and heterozygous mice). Maximum life span (mean life span of the oldest 10 % within a cohort) was decreased by 162 days (from 838 to 676 days). Data for females showed that the median life span in females TauTKO mice was decreased by 53 days (from 847 to 794 days) relative to that of controls, and female maximum life-span was also decreased by 60 days (from 948 to 888 days). These data indicate that tissue taurine depletion shortens lifespan in both sexes, with males being more affected. Gender-specific effects for lifespan have also been reported in other studies. For instance, the lifespan extension by target disruption of ribosomal protein S6 kinase 1 (S6K1) or treatment of rapamycin, an inhibitor of mammalian target of rapamycin, was observed only in female mice (Harrison et al. 2009; Selman et al. 2009). The pathways involved in these molecules might be related to the molecular mechanisms underlying shortening of lifespan in TauTKO mouse.

2.2 Cardiac Aging

We have reported that the myocardium of young knockout mice exhibited histological abnormalities, such as dilated ventricles and reduction in ventricular thickness (Ito et al. 2008). In addition, cardiac marker genes, including atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and beta-myosin heavy chain (βMHC), were

induced in TauTKO heart. Importantly, reduction of these genes was more enhanced in old TauTKO mice than young mice. Cardiac function, assessed by echocardiographic analysis, was deteriorated in old TauTKO mice (more than 9-month-old), but not in young TauTKO mice. Therefore, cardiac taurine depletion predisposes mice to chronic heart failure and aging accelerates this functional disorder.

2.3 Skeletal Muscle Aging

Both, we and another group, have reported that exercise endurance capacity is decreased in young TauTKO mice (Ito et al. 2008; Warskulat et al. 2004). We further found that skeletal muscle taurine loss leads to skeletal muscle atrophy and ultrastructural abnormalities, such as myofilament fragmentations, membranous cytoplasmic bodies and lipid droplets. Therefore, these histological abnormalities may lead to the reduction of exercise performance.

In old TauTKO muscle (more than 18 months old), myotubes with center nucleus are frequently observed compared to WT muscle. While nuclei are usually located around myotubes but not in center of cells, central nucleus is a feature of regenerating muscle fiber. This is well observed in aging muscle as well as in the dystrophic muscle (Ito et al. 2014a). The reduction of electron transport chain complex 1 activity, another marker of aged muscle, is also detected in old TauTKO muscle.

To explore the direct effect of taurine depletion on cellular senescence, we analyzed the levels of p16 in various tissues of aged male TauTKO mouse. Cyclin kinase inhibitor p16INK4a is well known biomarker of aging. P16 INK4a is a cell-cycle regulatory protein that interact with CDK4 and CDK6, inhibiting the ability to interact with cyclin D. Thereby, p16 inhibits cell-cycle and cell proliferation. It has been demonstrated that expression of p16INK4a markedly increases in almost all rodent tissues with advancing age (Krishnamurthy et al. 2004). Recently, it has been reported that clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders (Baker et al. 2011), illustrating the critical role of p16. P16 in skeletal muscle was higher by more than tenfolds in old (18–22-month-old) TauTKO than old WT mice. The expression of p16 in lungs and kidneys was also higher in old TauTKO but the increase was lesser than muscle. In contrast, it was not changed in heart, liver. Therefore, skeletal muscle is susceptible to cellular senescence mediated by tissue taurine depletion.

2.4 Aging Effect on Skin Wound Healing

Delays in cutaneous wound healing are another important feature of aging. To evaluate wound healing, we determined the degree of wound healing in TauTKO and littermate WT mice (Fig. 1). Skin excisions were prepared by 8 mm biopsy punch and then monitored for 1 month. In old mice (12-month-old), several days after

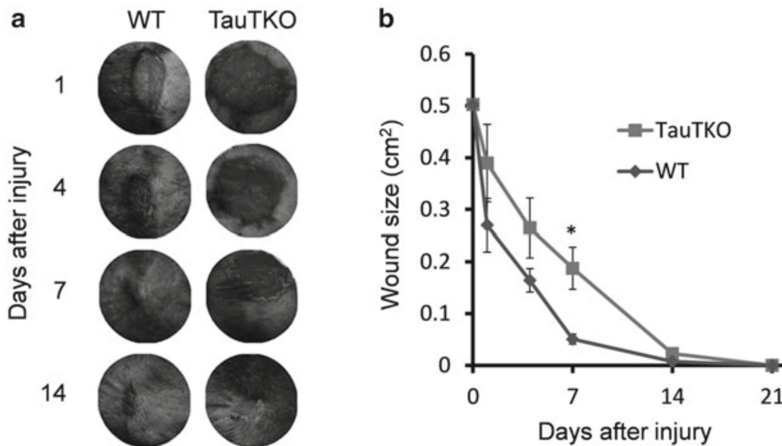

Fig. 1 Cutaneous wound healing in old TauTKO mice. After wound was created by 8 mm biopsy punch, wound size was monitored for 3 weeks in 12-month-old WT and TauTKO mice. n = 5. Representative photographic images (**a**) and calculation of wound size (**b**) indicate the significant delay in wound healing in TauTKO mice. *$p < 0.05$ vs. WT

biopsy a significantly greater reduction in wound closure was observed in TauTKO mice compared to WT mice. In contrast, we failed to detect difference in the speed of wound healing between young TauTKO and WT mice (5-month-old). Therefore, wound healing may also be age-dependent.

2.5 *Hepatic Aging*

Warskulat et al. (2006) have reported that moderate hepatitis and hepatic fibrosis were frequently detected in TauTKO mice than WT mice beyond 1 year of age, but not at young age (Warskulat et al. 2006). This hepatitis in old TauTKO mice was characterized by hepatocyte apoptosis, activation of the CD95 system, elevated plasma TNF-α levels, indicating a state of chronic inflammation. Moreover, the enhanced hepatic stellate cell and oval cell proliferation were detected in histological analysis, suggesting that the proliferative capacity of residual TauTKO hepatocytes is impaired and insufficient to counteract the hepatocyte loss.

3 Taurine as Proteostasis

In general, several organic osmolytes, such as taurine and other amino acids, which are also referred to as “compatible” osmolytes are known to regulate cell volume against osmotic alterations and to stabilize protein structures. While some inorganic

ions generally bind to proteins, which in turn perturbs proper protein folding, many compatible osmolytes exclude these effects from protein surface (Burg and Ferraris 2008; Yancey 2005). Moreover, organic osmolytes maintain the intracellular concentration of inorganic ions. For instance, when cells are exposed to hypertonicity, organic osmolytes are accumulated following acute elevation of inorganic ions, which associates with a decrease in concentration of intracellular inorganic ions.

The role of taurine as well as other compatible osmolyte on the protein folding has been confirmed in the mutant cystic fibrosis transmembrane conductance regulator (CFTR) protein (delta508 CFTR) (Howard et al. 2003). The deletion of a phenylalanine residue at position 508 of CFTR (deltaF508CFTR) causes cystic fibrosis. This mutation results in decreasing the trafficking of protein to the cell surface due to failure of proper folding. Treatment of taurine in delta508CFTR-expressing cells was effective to proper folding and processing of this protein. More recently, it has been reported that taurine was effective in improving the folding of Fab in the process of artificial human Fab production from recombinant human Fab-H chain and L chain (Fujii et al. 2007). Notably, it has been reported that taurine treatment attenuates ER stress induced by homocysteine in cultured vascular endothelial cells (Nonaka et al. 2001), suggesting that taurine ameliorates the accumulation of misfolded protein in homocysteine-treated cells.

Meanwhile, we investigated the molecules which are involved in unfolded protein response (UPR) in TauTKO muscle to explore whether taurine depletion impairs proper protein folding (Ito et al. 2014a). It is assumable that impairment of protein folding by cellular taurine depletion leads to accumulation of misfolded proteins and triggers ER stress. According to the results of microarray analysis which compare genes expression between TauTKO and WT mouse, heat shock proteins, such as Dnaja4, Hspa1a, Hspa1l, Hspa5 (BiP) and Hspb7, and transcriptional factors involved in UPR, such as XBP1, ATF3 and Crem, are significantly increased in TauTKO tissue. Further, experiments indicate that BiP was increased in TauTKO muscle at the protein level. Moreover, nuclear content of spliced form of XBP1 protein was also increased in TauTKO muscle. Therefore, impairment of protein folding by taurine loss may result in the activation of protein homeostasis, including heat shock proteins and unfolded protein response, to compensate for stress.

4 Conclusion

In conclusion, these data suggest that endogenous taurine acts as an anti-aging molecule in several tissues. Especially in skeletal muscle, taurine loss leads to early onset of cellular senescence. It must be noted that the other taurine actions, anti-oxidation and calcium handling, may also contribute to protein stability, since oxidative stress injures proteins and impairment of calcium homeostasis perturbs proper protein folding in endoplasmic reticulum. Meanwhile, the effect of taurine treatment in healthy animals or human on the lifespan or tissue senescence remains to be clarified. The finding that tissue taurine loss accelerates muscle senescence

implies the possibility that taurine treatment can protect skeletal muscle from aging. Future studies are necessary to determine whether taurine treatment delays muscle senescence and in turn extends not only lifespan but also healthspan.

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Analysis of Taurine as Modulator of Neurotransmitter in *Caenorhabditis elegans*

Hyunsook Chang and Dong-Hee Lee

Abbreviations

GABA	γ-Aminobutyric acid
GAD	Glutamic acid decarboxylase
NGM	Nematode growth media
SR	Success ratio

1 Introduction

Taurine is synthesized from methionine throughout the human body, and from cysteine in the liver in the presence of vitamin B6. As it is stored in various types of cells, taurine plays an important role in maintaining homeostasis by sustaining an osmotic balance within animal cells. It helps cells recover from damage and prevents physiological stress by preserving osmolarity. In neuronal cells, taurine is involved in intercellular ion trafficking via functional regulation of the ion channels. Taurine plays an important role in the development, survival, and neural growth of vertebrate neurons. Along with caffeine, taurine is one of the most utilized psychoactive drugs and serves as a psychoactive agent that can adjust perception, mood, consciousness, or behavior. Since taurine is commonly applied in combination with

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caffeine, the psychoactive effect of taurine alone is difficult to evaluate Whirley and Einat (2008) have even negated taurine's psychoactive effect. They claim that taurine's psychoactive attributes are misleading since their animal study failed to confirm any specific improvement using taurine.

When treated with taurine, the mouse brain responds with significant changes in the γ-aminobutyric acid-producing (or GABAergic) neurons (El Idrissi 2008). Taurine induces hyperexcitability by lowering the levels of $GABA_A\beta$ subunits and augmenting the expression of glutamic acid decarboxylase (GAD) and GABA, most likely due to the sustained interaction of taurine with $GABA_A$ receptors. This can be considered a potential mechanism for coping with taurine administration, and it can affect motor behavior (Niebur and Erdos 1993; Santora et al. 2013). Taurine's effects may stem from its structural similarity to GABA. Still, taurine's function as a psychopharmaceutical or psychotropic agent is highly controversial among taurine researchers. Although its psychostimulative effect has been attested in numerous human studies, Giles and colleagues (2012) state that mental enhancement by taurine can be achieved only in the presence of caffeine.

The act of learning typically occurs in the early stages of development in most animal species. It is a sophisticated process of adaptation or classical habituation, rather than a simple gathering of actual and practical information (Vallotton and Ayoub 2010; Wicks and Rankin 1997). Among the various types of learning, associative learning refers to having a new response attached to a particular stimulus in animal behavior (D'Adamo et al. 2004; Wen et al. 1997). This term covers almost all types of learning, except simple acclimatization. Despite lacking a central nervous system, recent reports indicate that *Caenorhabditis elegans* exhibits a basic form of associative learning (Amano and Maruyama 2011; Pandey et al. 2011; Qin and Wheeler 2007). *C. elegans* has short-term and long-term memory and behavioral flexibility related to learning, both associative and non-associative (Amano and Maruyama 2011; Ardiel and Rankin 2010).

C. elegans is practical for studying various biological functions despite its simple structure. It has become a prized animal system for furnishing an integrated view of organismal responses to numerous forms of environmental stimulation (Croll 2009). In combination with the comprehensive genetics and neuroanatomy of *C. elegans*, some of its mutants show deficiencies in associative learning, thus providing the foundation for an actual characterization of the cellular and molecular aspects of associative learning.

C. elegans has a nervous system consisting of 302 neuronal cells. This number is remarkable considering a wild-type adult body consists of 959 somatic cells (Albeg et al. 2011). In the nematode, neuronal synapses are found mostly in the nerve ring, ventral nerve cord, and dorsal nerve cord (Lee et al. 2012). The nerve ring is the brain of the worm and the complex behaviors of the worm are coordinated in this area. Despite having only 5,000 synapses among the 302 neurons, *C. elegans* displays complex forms of behavior including habituation, sensitization, and conditioning in response to stimuli such as drugs and neurotransmitters. *C. elegans* has a particular set of proteins to synthesize, transport, or utilize GABA, and these proteins

are required for its neurotransmission; the proteins include a biosynthetic enzyme, vesicular transporter, transcription factor for shaping GABA neuron identity, and different receptors for inhibition and excitation (Schuske et al. 2004). This makes it a functional system for understanding behavioral plasticity in terms of its neurochemistry, the neurobiology of potential neurotransmitters, and evolutionally conserved molecules such as insulin, monoamines, and neuropeptides (Lorimer et al. 1996; Rankin et al. 1990; Sasakura and Mori 2013). When compared to mammalian neurons, the neurons of *C. elegans* are remarkably similar in terms of their essential functions and connectivity, in spite of its simplistic nervous system.

To characterize the potential effect of taurine on associative learning, groups of *C. elegans* were treated with taurine or caffeine in the present study. Caffeine is a highly used psychoactive drug that is often used with taurine as a co-stimulant in many types of energy drinks (Zeratsky 2008). Although caffeine extends the life span of *C. elegans*, its effect on learning has not been studied in the nematode (Sutphin et al. 2012). In vertebrates, behavioral plasticity that leads to learning is assessed using mazes, and recent studies have shown that a similar approach can be applied to nematodes using microfluidic channel techniques (Chronis 2010; Crane et al. 2010; Lockery et al. 2008). However, without interdisciplinary efforts, a microfluidic maze is hard to prepare (Park et al. 2008; Rohde et al. 2007).

In this study, a new type of maze was invented by placing coverslips on an agar gel. Gaps can be easily generated and worms can move along the path created by the gaps. The effect of taurine was studied by monitoring the difference in the success ratio (SR) of foraging between the taurine-treated and taurine-free groups. The effect of taurine on movement was also studied by comparing the distance traveled between the taurine-treated nematodes and their non-treated counterparts. The aim of the present study was to address taurine's effect on the nematodes' initial foraging abilities and the enhancement of learning attached to foraging, which refers to looking or searching for food.

2 Methods

2.1 Strain and Culture Conditions

The N2 strain of *C. elegans* was maintained under typical conditions (Stiernagle 2006). When treated with taurine or caffeine, *C. elegans* grew in S medium having concentrated *Escherichia coli* OP50 and the respective treatment agents. Taurine was added into the OP50 mixture to the final concentrations of 5 and 10 mg/mL in the S media. For the control worms, no taurine supplementation was provided when treated in the S media. In addition to taurine, worms were also treated with caffeine at 0 or 5 mg/mL as the final concentration. Therefore, worms were treated with six different combinations of taurine-caffeine concentrations: 0-0, 0-5, 5-0, 5-5, 10-0, and 10-5 (mg/mL).

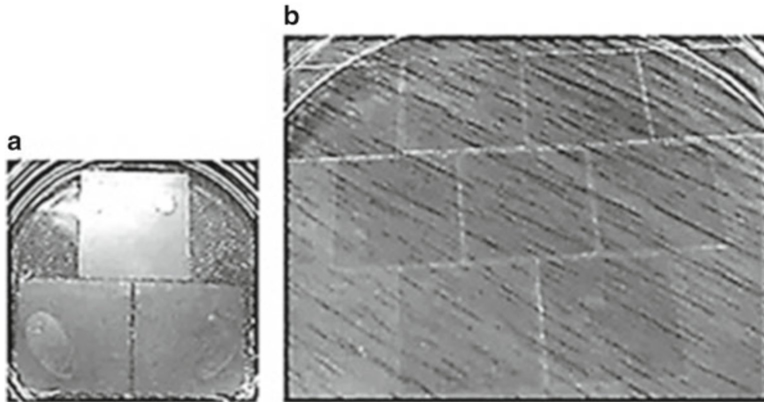

Fig. 1 Simple and complex mazes. The mazes were assembled by placing microscopic coverslips on the surface of agarose gel. Gaps were made between the coverslips, and their width was approximately 1 mm. The height was 0.5 mm and the worms were unable to climb up the edge. The simple maze (**a**) consisted of three coverslips, while the complex maze (**b**) was assembled using nine coverslips

2.2 Maze Construction

After overnight growth with taurine or caffeine in OP50, worms were thoroughly washed in M9 media. They were then transferred to the simple maze, which was made by utilizing gaps between pieces of microscopic coverslips, as shown in Fig. 1. Two types of mazes were used: simple (T-shaped) and complex (branched). The edges of the coverslips were mildly coated with petroleum jelly (Vaseline); otherwise, worms would stick to the edge by extending their bodies. The coverslips were arranged so that they were in close contact with the surface of the nematode growth media (NGM) gel, without air bubbles or gaps between the coverslips and the gel. Once worms were placed at the start inlet, the area was sealed using Vaseline ointment to prevent the worms from moving out of the maze.

2.3 Calculation of Success Ratio and Comparison

The worms were allowed to move along the maze to forage until they reached the end of each maze. In the simple maze, one end had an area of OP50, with the other area being free of OP50. In the case of the complex maze, only one outlet had the food, while the other two ends did not have food. The worms were subjected to the maze experiments for 3 days before the data were analyzed. Each foraging success was compared among the six different taurine-caffeine concentrations. At each end, worms were counted and the success ratio (SR) was calculated as the

percentage of worms found at the food area per the total number of participating worms. The SRs were compared between the taurine and taurine-free treatments. The SR was calculated by the following formula: SR (%) = number of successful worms/number of total participating worms × 100. Comparisons were made in terms of the SR among the different treatments according to the analysis of variance (*ANOVA*) or Student's *t*-test.

2.4 Movement Comparison Assay

Another group of worms was placed on the complex maze and the SR was calculated using the same procedures as those described above for the simple maze. In addition to the SR, taurine-treated and taurine-free worms were compared in terms of the distance traveled on the gel. Ten worms were permitted to move on the gel for 24 h, and the total distance traveled was visually quantified and compared. The distance traveled by the worms was divided into five groups and each grade of length was scored as in the number of plus (+) symbols.

3 Results

Using the *C. elegans* system, this study focused on whether the worms' foraging abilities could be accelerated following treatment with taurine. The effects of taurine on learning were assayed alone or in combination with caffeine. Taurine treatment alone significantly augmented the ability of the worms to find food. Worms that underwent taurine treatment appeared to have an accelerated learning ability compared to the control worms. In combination with caffeine, taurine helped worms forage successfully in the case of the complex maze. Worms showed significant improvements in reaching the area with food when treated with both taurine and caffeine.

3.1 Taurine Increases Foraging Success Ratios for the Simple Maze

Worms were treated with taurine or caffeine in the liquid S media containing each treatment chemical. Worms were subjected to the maze experiments for 3 days to analyze their foraging performance after taurine treatment. When taurine-treated worms were placed in a simple maze and allowed to move freely to find the area with food, differences were apparent between the treatment groups. The taurine-treated worms showed notable success on the simple maze in terms of their foraging performance when the SR was compared among the six different combinations of

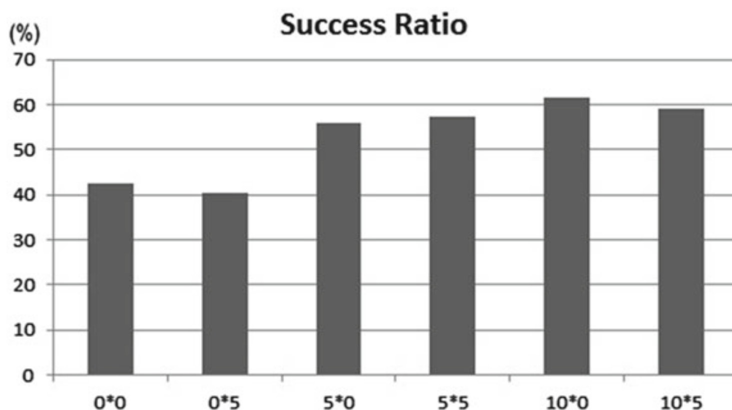

Fig. 2 Success ratios (SRs) for the simple maze

taurine and caffeine following the treatments (Fig. 2). More taurine-treated worms appeared to reach the OP50 area compared to the taurine-free control group. No significant differences were apparent between the taurine only and taurine-caffeine co-treated groups. When the worms were treated only with caffeine (5 mg/mL) and subjected to the simple maze, their SRs were significantly lower than the SRs of the taurine-only treatment group. This shows that caffeine alone exerted a minimal effect in the case of the simple maze experiment.

Groups of *C. elegans* were treated with taurine or caffeine (six different concentrations) and placed into the simple maze. After 3 days, the majority of worms reached either end of the T-shaped maze ($n=30$). Taurine-treated worms show a higher ratio of success in reaching the arm with the OP50 ($p<0.05$). As for the different taurine concentrations, worms appeared to increase the foraging SR. The X-axis refers to the concentrations of taurine (t) and caffeine (c) in terms of mg/ mL.

3.2 Foraging Success Increases in the Presence of Caffeine for the Complex Maze

To examine the effects taurine on foraging success in the complex maze, worms were pre-treated with taurine and subjected to the complex maze experiments. When the SRs were measured after 3 days, the taurine-treated group was more successful at reaching the OP50 area compared to the taurine-free control group (Fig. 3). This result is consistent with the outcome from the simple maze study. When treated with caffeine in addition to taurine, however, worms appeared to show increased SRs, which was different from the results of the simple maze experiment.

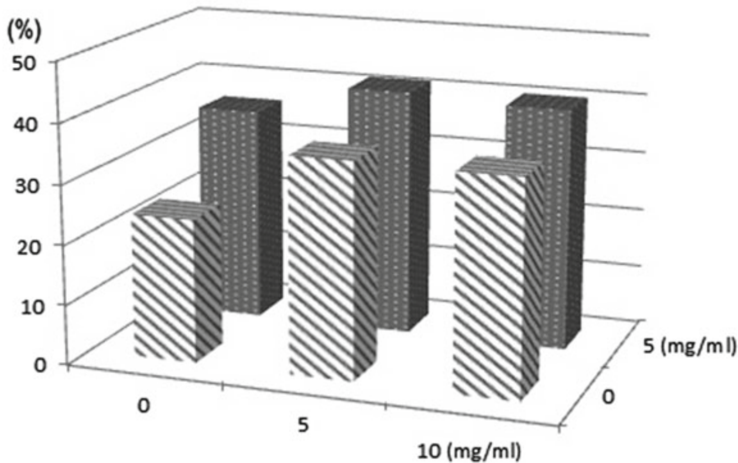

Fig. 3 Success ratios (SRs) for the complex maze

The effect of taurine on foraging performance was assayed using the complex maze. Worms were pre-treated with taurine and subjected to the complex maze experiments. The SRs were compared after 3 days between the three different treatment groups. When treated with caffeine in addition to taurine, worms show increased SRs, which is different from the results of the simple maze experiment ($p<0.05$). The concentrations at front and side belong to taurine and caffeine, respectively. The SRs are lower than 50 % since the complex maze has three outlets, different from the case of the simple maze that has no more than two outlets.

3.3 Repeated Application of Taurine Augments Foraging Success for the Simple Maze

Worms that demonstrated successful foraging were collected and divided into two groups. One group of worms was briefly treated with taurine and the other was not. Both groups were subjected to the simple maze experiment and their performances were compared. A difference was evident between the two groups (Fig. 4). The taurine-treated group showed a higher SR in comparison to the taurine-free group. A similar experiment was performed using the complex maze. No differences were shown among the taurine-treated and taurine-free groups (data not shown).

Successful worms were treated further with taurine and caffeine on media. The control group was not treated with taurine. Then, the worms were subjected to the simple maze. In terms of the SR, the taurine-treated group showed a higher SR compared to the taurine-free group ($p<0.05$). The X-axis refers to the concentrations of taurine (t) and caffeine (c). The light and dark bars represent non-treated

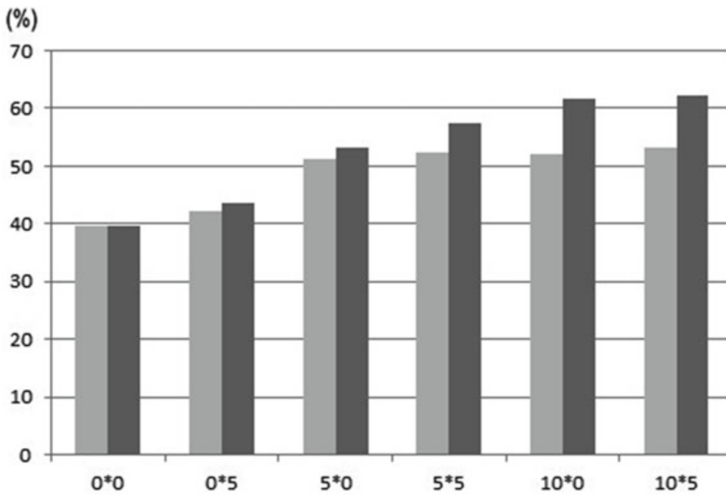

Fig. 4 Simple maze experiment on worms with successful foraging

and treated groups, respectively, except for [0*0]. Using the complex maze, a similar experiment was performed to compare the SRs between the taurine and taurine-free treatments. There were no apparent differences between the taurine-treated and taurine-free groups (data not shown).

3.4 Combination of Taurine and Caffeine Promotes Motility of C. elegans

The potential effects of taurine and caffeine on *C. elegans* were also assayed in terms of motor activity. Along with the increased foraging success, the distance traveled by the worms increased when treated with taurine. The total movement of the worms was visually compared between the taurine-treated and taurine-free groups. When treated with taurine, worms appeared to travel farther than their non-treated counterparts (Table 1). Comparisons were also made between the caffeine and caffeine-free taurine treatments. The distances traveled by the worms increased when treated with caffeine. Increased movement was evident for the worms treated with caffeine in addition to taurine. This strongly indicates that taurine has a positive effect on the worms' motor activity.

The length of the path that worms took on the gel surface was visually compared after each treatment. The length was quantified into five levels. The taurine-treated group showed an increase in the distance traveled compared to the taurine-free controls. The length of the movement paths appeared to increase in a concentration-dependent manner.

Table 1 Visual comparison of the distances traveled

Taurine (mg/ml) Caffein (mg/ml)	0	5	10
0	+	+++	++++
5	++	+++	+++++

4 Discussion

In the present study, *C. elegans* showed a significant level of success in searching for food when treated with taurine. Taurine clearly helped the worms find the area with food or to successfully forage for food. When treated with caffeine in addition to taurine, however, worms showed better responses via increased SRs, especially in the complex maze study. Taurine produced more positive effects when combined with caffeine.

In vertebrates, GABA serves as a vital neurotransmitter that works at synapses in the central nervous system. In *C. elegans*, however, GABA works mainly at neuromuscular synapses to ease the body muscles during movement and foraging. Because taurine resembles GABA in structure, taurine is expected to work similarly in the muscles. One possible explanation for the increased foraging SRs may be related to a taurine-mediated increase in the total distance traveled, that is, the increased motor activity and distance traveled may result in the rates of successful foraging on a trial and error basis.

Foraging has been perceived as the primary subject of associative learning. Trial and error is essential for the development of foraging since the worms learn how to cope with their environment. For *C. elegans*, vigorous locomotive movement may help them find their food source when they are treated with taurine or caffeine. After treatment with taurine, *C. elegans* showed increased travelling distances and flexibility; thus, augmented locomotive activity improves the chance for successful foraging.

When successful worms were subjected to the simple maze experiment, they showed a higher rate of success under a repeated application of taurine. Despite the brief application of taurine, the worms showed significant SRs for foraging. In the taurine-free group, the level of success was lower. This strongly indicates that taurine may help worms improve their foraging. In the case of the complex maze, however, no difference was evident between the taurine and taurine-free groups. This result indicates that taurine may not work sufficiently toward associative learning due to the simplicity of the worm's nervous system.

Taurine is known to shorten the reaction time in working memory tasks, although it extends decision-making time for humans. Caffeine, however, helps improve executive control and working memory, and reduces simple reaction and decision-making times (Giles et al. 2012). In this study, caffeine was highly functional in the complex maze study. This result might come from the fact that caffeine helps reduce the length of time required for decision-making. In the simple maze, worms experienced once to be successful in foraging; however, they had to encounter at least

twice to finalize the appropriate foraging. For this doubled case of decision-making, caffeine might have provided a "correct" decision twice for successful foraging, and thus its effect became noticeable in this study. Decision-making usually involves the ability to assess the significance of obtainable choices from experience. Taurine might have played a role in associative learning. Furthermore, taurine might have helped the worms adjust their behaviors for an optimal foraging task as an associative learning model.

This study suggests that taurine may work alone to exert a convincing effect on associative learning in the absence of caffeine. This strongly indicates that taurine affects worms in a positive way during the course of associative learning. Caffeine provides a synergistic effect on associative learning when combined with taurine. Future studies may be necessary to describe the degree of synergy between taurine and caffeine for enhancing the level of associative learning. Additionally, a future study should employ locomotion to verify the results of the present study.

5 Conclusion

This study focused on whether the foraging abilities of the *C. elegans* could be promoted upon treatment with taurine alone or in combination with caffeine. Taurine alone considerably increased the capability of the worms to find food and promoted learning ability compared to the control worms. This observation proposes that taurine may work alone to exert a definite effect on associative learning in the absence of caffeine. In combination with caffeine, worms showed substantial progresses in reaching the area of food. This strongly indicates that taurine alone affects worms in a positive way during the course of associative learning and that caffeine provides a synergistic effect on associative learning when combined with taurine.

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The Changes by Hypoxia Inducible Factor-1alpha (HIF-1α) on Taurine Uptake in Brain Capillary Endothelial Cells at High Glucose Conditions

Na-Young Lee and Young-Sook Kang

Abbreviations

2ME2	2-Methoxy estradiol
ALD	Alendronate
BA	Betulinic acid
BBB	Blood-brain barrier
HIF-1	Hypoxia inducible factor-1
MTX	Methotrexate
TAUT	Taurine transporter
VEGF	Vascular endothelial growth factor
YC-1	3-(5′-Hydroxymethyl-2′-furyl-1-benzylindazole)

1 Introduction

Taurine, a free sulfonic acid, acts as a neuromodulator, a neuroprotector, an antioxidant, and an anti-inflammatory agent (Foos and Wu 2002; Pan et al. 2010; Sun et al. 2011). Several studies suggest that taurine also can prevent the hyperglycemia-induced the microvascular complications of diabetes, such as neuropathy, retinopathy, and autonomic dysfunctions (Ito et al. 2012). The taurine supply to the brain from the circulating blood is mediated by Na^+, Cl^--dependent taurine transporter, TAUT, in brain microvascular endothelial cells (blood-brain barrier, BBB) (Kang et al. 2002). This TAUT at the BBB is involved in the maintenance of taurine levels in the brain. In hyperglycemia, elevated glucose is believed to contribute to loss of

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microvascular barrier integrity (Lorenzi et al. 1986; Frank 2004). Accordingly, transport of taurine at BBB may be also changed by hyperglycemia, and this change could intensely affect the neuroprotective effect of taurine by influencing taurine concentration in the brain. In previous studies, it has been reported that TAUT was regulated by glucose, oxidative stress, and changed in osmolarity (Stevens et al. 1999; Kang et al. 2002; Kang et al. 2009). However, the mechanisms involved in the change of taurine transport remain unclear. Hypoxia inducible factor 1 (HIF-1) is an important factor of diabetes neuropathy, and up-regulates vascular endothelial growth factor (VEGF) (Forsythe et al. 1996), which is well defined in promoting new blood vessel formation and causes BBB disruption and increases BBB permeability (Schoch et al. 2002; Yeh et al. 2007). High glucose has been reported to up-regulate HIF-1 activity in isolated hearts of rats (Marfella et al. 2002) and kidney mesangial cells (Isoe et al. 2010). However, it is not known if HIF-1 is involved in change of taurine transport in brain endothelial cells exposed to high glucose. The purpose of the present study was to determine whether or not HIF-1 played a role in taurine transporter activity at high glucose condition. The study was carried out with an in vitro BBB model of conditionally immortalized rat brain microvascular endothelial cells (TR-BBB cells). Previous publications have well characterized TR-BBB cells as an appropriate model of in vitro BBB, which has been shown to express a number of transporters that are expressed in endothelial cells comprising the BBB, allowing for the characterization of their functions in vitro (Hosoya et al. 2000; Lee and Kang 2010).

2 Methods

2.1 Cell Culture

The TR-BBB cells were cultured according to the previous report (Lee et al. 2012). The TR-BBB cells were seeded at 1×10^5 cells/well in rat tail collagen type 1-coated 24 well culture plates (Iwaki, Tokyo, Japan) for the uptake study. After incubation for 2 days at 33 °C, the cultures became confluent and then were used in uptake study.

2.2 [^{3}H]Taurine Uptake Study in TR-BBB Cells

The [^{3}H]taurine uptake was performed according to the previous report (Lee et al. 2012). Briefly, cells were washed three times with 1 mL extracellular fluid (ECF) buffer. Uptake was initiated by addition of ECF buffer containing [^{3}H]taurine (28 nM) and then incubate at 37 °C for 5 min. [^{3}H]Taurine uptake into the cell was terminated by the addition of ice-cold ECF buffer. The cells were then solubilized in 1 N NaOH, and radioactivity was measured in a liquid scintillation counter (LS6500; Beckman, Fullerton, CA). To investigate the change of [^{3}H]taurine uptake under high glucose condition, the TR-BBB cells were pretreated with 25 mM glucose for 12, 24, 36 and 48 h and the uptake study was performed as described above. To test the effect of HIF-1 and VEGF inhibitors on the change of [^{3}H]taurine uptake under

high glucose condition, TR-BBB cells were exposed to 10 μM 2-methoxy estradiol (2ME2), 10 μM 3-(5′-hydroxymethyl-2′-furyl-1-benzylindazole) (YC-1), 10 μM betulinic acid (BA), 10 μM alendronate (ALD) and 10 μM methotrexate (MTX) for 1 h under exposing TR-BBB cells to 25 mM glucose for 48 h. Cell to medium ratio (μL/mg protein) was calculated as follows:

Cell to medium ratio (μL/mg protein) = ([^{3}H] dpm in the cell/amount (mg) of cell protein)/([^{3}H] dpm in the injectate/amount (μL) of injectate) × 100.

2.3 Real-Time Reverse Transcription Polymerase Chain Reaction

Total RNA was isolated from cultured TR-BBB cells by the RNeasy kit from Quiagen (Quiagen, Valencia, CA) and according to the manufacturer's instructions. Total RNA (2 μg) was reverse-transcribed by using oligo (dT) and high capacity RT kit (Applied Biosystems, Foster City, CA). Quantification of TAUT mRNA was performed on an ABI 7500 Sequence Detector System (Applied Biosystems, Foster City, CA). 5 μL of complementary DNA (cDNA) were used for real-time PCR. Gene-specific oligonucleotide primers and probes for TAUT, HIF-1α, VEGF as well as the endogenous control β-actin were obtained from Applied Biosystems. The reaction contained 10 μL TaqMan Master Mix (Applied Biosystems, Foster City, CA) and 1 μL of the specific primer in a final volume of 20 μL. Reaction condition were 30 min at 48 °C, 10 min at 95 °C, 40 cycles for 15 s at 95 °C and 1 min at 60 °C, and a final 5 min incubation at 25 °C.

2.4 Immunocytochemical Staining

First, we quenched endogenous peroxidase activity in TR-BBB cells by incubating tissue for 30 min in Thermo Scientific Peroxidase Suppressor and then TR-BBB cells were washed with PBST. This step was followed by blocking buffer for 30 min, which we have found to be an effective blocker of nonspecific binding. Cells were incubated with the primary antibody [TAUT C-15 (Santa Cruz Biotechnology, Santa Cruz, CA)] in PBST for 60 min at room temperature. Following washes three times for 10 min each with PBST, cells were incubated with HRP-labeled secondary antibody for 30 min. And then we washed cells three times for 10 min each with PBST. Cells were added metal enhanced DAB solution and incubated for 5 min. After washing the cells two times for 3 min each with PBST, cells were examined under a light microscope.

2.5 Statistic Analysis

Statistical significance was determined by one-way ANOVA with Dunnett's post-hoc test. Each value was expressed as the mean ± SEM. Differences were considered statistically significant when the calculated P value was less than 0.05.

3 Results

3.1 Effect of High Glucose on the [^{3}H]Taurine Uptake in TR-BBB Cells

To investigate the effect of excess glucose on taurine uptake at the BBB, [^{3}H]taurine uptake activity was examined in TR-BBB cells at high glucose pre-treatment conditions. In this study, the glucose level of 25 mM was used as high glucose condition against 5.5 mM as normal control. By exposing TR-BBB cells to high glucose for 48 h, [^{3}H]taurine uptake was decreased continuously (Fig. 1).

3.2 Effect of High Glucose on HIF-1α and VEGF mRNA Expression in TR-BBB Cells

HIF-1 is a heterodimer that is composed of α and β subunits. It has been known that the HIF-1α level primarily determines HIF-1 activity (Wang et al. 1995). Therefore, we investigated the change of mRNA levels of HIF-1α and VEGF in TR-BBB cells under high glucose condition. As shown in Fig. 2, high glucose induced HIF-1α and VEGF mRNA expression. Increased HIF-1α and VEGF mRNA expression was effectively reduced by 2ME2 as HIF-1 inhibitor and MTX as VEGF inhibitor, respectively (Fig. 2).

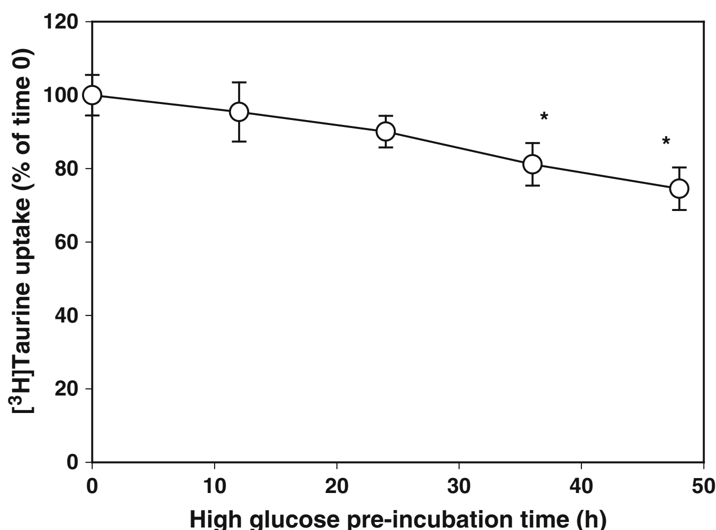

Fig. 1 Change of taurine uptake under high glucose condition by TR-BBB cells. 25 mM glucose was pre-incubated for the time period in the figure. The cells were incubated for 5 min at 37 °C with ECF buffer containing [^{3}H]taurine (28 nM). Each point represents the mean ± SEM (n = 4). *$p < 0.05$; significantly different from time 0

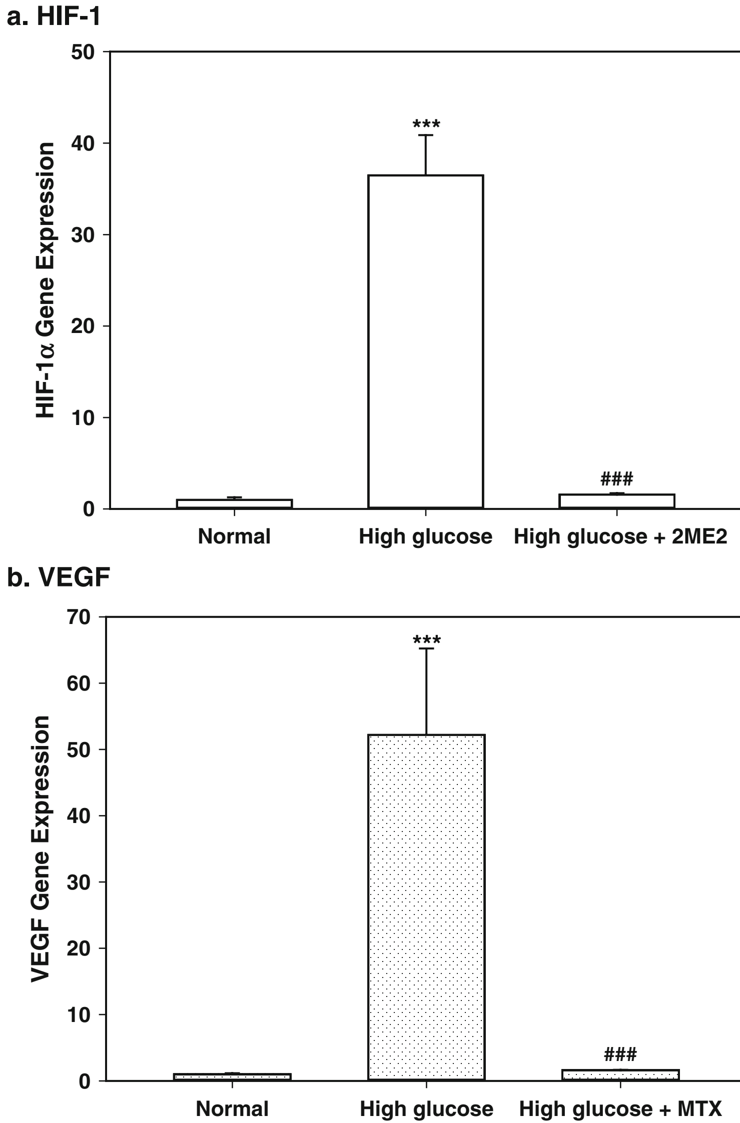

Fig. 2 Change of HIF-1α (**a**) and VEGF (**b**) mRNA expression under high glucose condition by TR-BBB cells. Cells were pre-treated with glucose at 5.5 (normal) and 25 mM (high) for 48 h. HIF-1 inhibitor (10 μM 2ME2) or VEGF inhibitor (10 μM MTX) were added to culture medium 1 h before the onset of high glucose treatment. Each bar represents the mean ± SEM (n = 3). ***$p < 0.001$; significantly different from normal control, $^{###}p < 0.001$; significantly different from high glucose

3.3 Effect of HIF-1 and VEGF Inhibitors on the [^{3}H]Taurine Uptake in TR-BBB Cells at High Glucose Condition

To further verify the role of HIF-1 and VEGF in high glucose induced taurine uptake decrease, we performed experiments with inhibition of HIF-1 and VEGF by HIF-1 inhibitors (2ME2, YC-1 and BA) and VEGF inhibitors (MTX and ALD). Both HIF-1 inhibitors and VEGF inhibitors induced [^{3}H]taurine uptake decrease caused by high glucose (Fig. 3).

3.4 Change of the Expression of TAUT Involved in HIF-1 in TR-BBB Cells at High Glucose Condition

Next, we tested whether glucose affected expression of taurine transporter, TAUT and the correlation between change of TAUT expression and HIF-1 in TR-BBB cells. TAUT gene expression was significantly reduced at 25 mM glucose, as

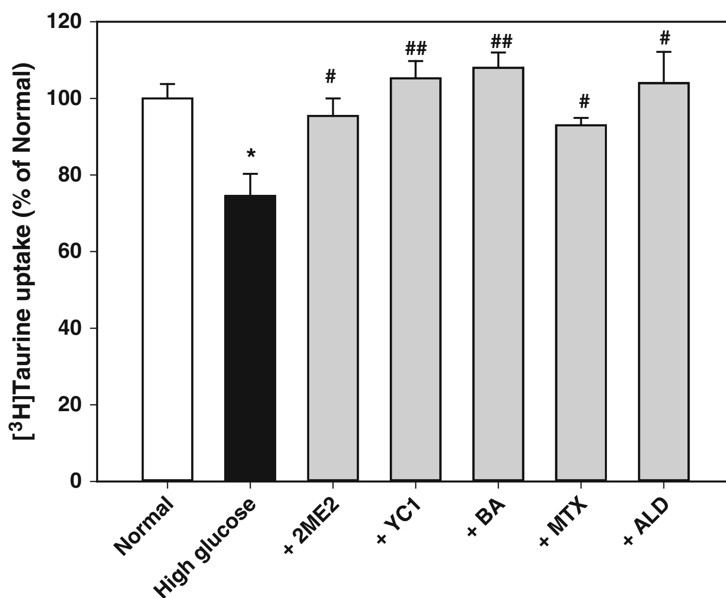

Fig. 3 Effect of HIF-1 and VEGF inhibitors on decreased [^{3}H]taurine uptake by high glucose in TR-BBB cells. The cells were pre-treated with 10 μM 2ME2, YC-1, BA, MTX and ALD for 30 min under exposing cells to 25 mM glucose for 48 h. [^{3}H]Taurine uptake was performed with ECF buffer containing [^{3}H]taurine (28 nM) for 5 min at 37 °C. Each point represents the mean ± SEM (n = 3). *$p < 0.05$; significantly different from normal control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$; significantly different from high glucose

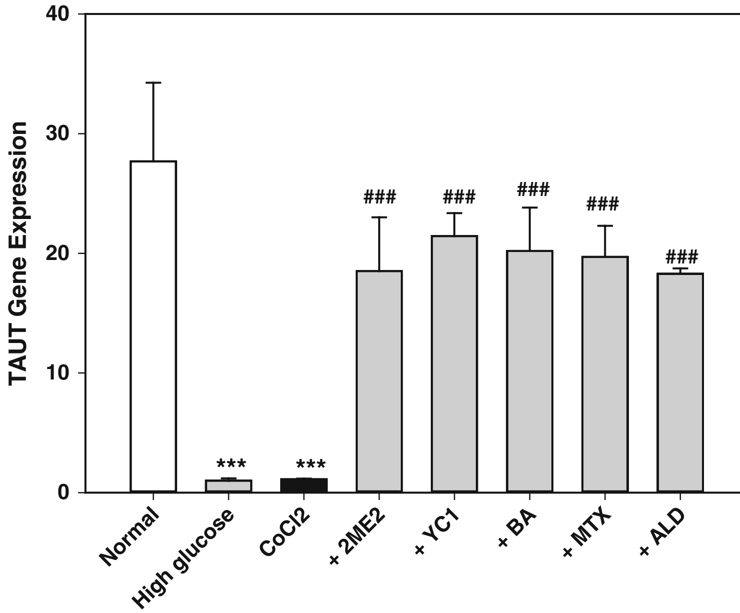

Fig. 4 Effect of high glucose (25 mM), HIF-1 inhibitors and VEGF inhibitors on TAUT mRNA expression in TR-BBB cells. Real time reverse transcription polymerase chain reaction (RT-PCR) analysis of TAUT mRNA expression in TR-BBB cells without and with pre-treatment with 25 mM glucose for 48 h or 100 μM $CoCl_2$ for 24 h. The cells were pre-treated with 10 μM 2ME2, YC-1, BA, MTX and ALD for 1 h under exposing cells to 25 mM glucose for 48 h. Each point represents the mean ± SEM (n = 3). ***p < 0.001; significantly different from normal control; $^{###}$p < 0.001; significantly different from high glucose

compared with that of normal control (Fig. 4). Also, TAUT protein level was decreased by pre-treatment 25 mM glucose (Fig. 5). These results were consistent with decrease of [^{3}H]taurine uptake caused by pre-treatment 25 mM glucose in TR-BBB cells. To determine the role of HIF-1 in high glucose induced decrease of TAUT expression, we performed experiments with up-regulation of HIF-1 in cells exposed to normal glucose. We used cobalt chloride as inducer of HIF-1 because cobalt chloride can inhibit HIF-1α degradation and increase HIF-1 activity (Yan et al. 2012). TAUT expression was significantly decreased by cobalt chloride, as compared with that of normal control (Figs. 4 and 5). The decrease of TAUT gene expression by pre-treatment 25 mM glucose was recovered by both HIF-1 inhibitors and VEGF inhibitors (Fig. 4). Also, HIF-1 inhibitor, 2ME2, induced the decrease of TAUT protein expression caused by high glucose (Fig. 5).

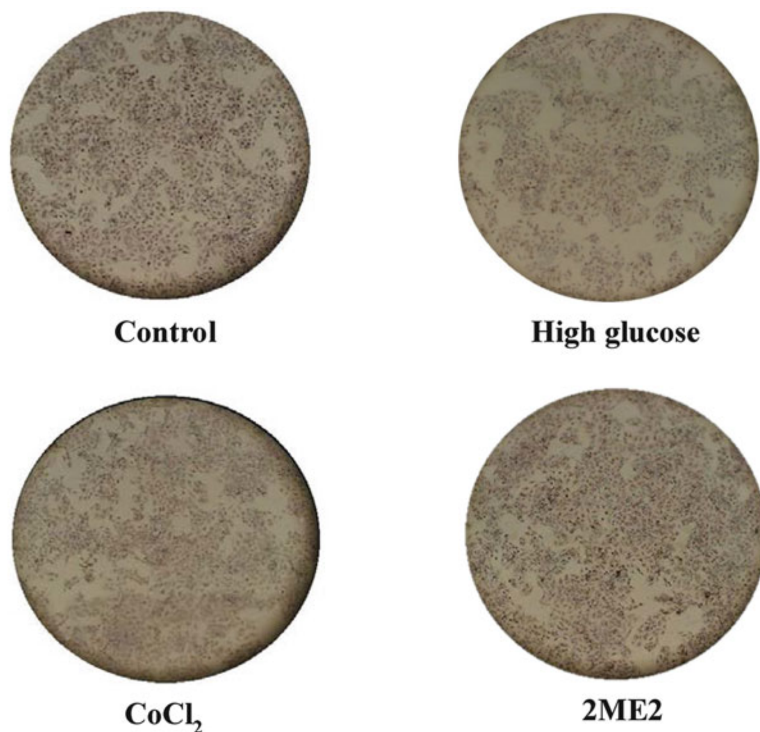

Fig. 5 Effect of high glucose and HIF-1 inhibitors on TAUT expression in TR-BBB cells. The cells were pre-treated without and with pre-treatment with 25 mM glucose for 48 h, 100 μM $CoCl_2$ for 24 h or 10 μM 2ME2 for 30 min under exposing cells to 25 mM glucose for 48 h. TAUT proteins in TR-BBB cells were immunostained with DAB

4 Discussion

In present study, we demonstrate that HIF-1 contributes to decrease of taurine transport at the BBB under high glucose condition. Actually, the changes in taurine transporter activities at high glucose condition have been reported in human retinal pigment epithelial cells (Stevens et al. 1999; Heller-Stilb et al. 2002). In our previous report, we clarified reduced taurine uptake in TR-iBRB cells as model of inner blood-retinal barrier under high glucose condition (Lee and Kang 2013). Our present result also revealed suppressed [^{3}H]taurine uptake in TR-BBB cells under high glucose condition for 48 h (Fig. 1). Until now, the mechanisms involved in the down-regulation of taurine transport remain to be unknown. HIF-1 is an important factor of diabetes neuropathy. It up-regulates VEGF, which causes BBB disruption and increases BBB permeability (Schoch et al. 2002; Yeh et al. 2007). Also, several evidences suggest that VEGF, and consequently angiogenesis, is related to the pathogenesis of diabetic retinopathy or neuropathy (Nicholson and Schachat 2010). Several reports have found that up-regulation of HIF-1 activity by high glucose in

isolated hearts of rats (Marfella et al. 2002) and kidney mesangial cells (Isoe et al. 2010). In vitro exposure to high glucose has been also shown to rapidly increase VEGF expression in various cell types and tissues (Natarajan et al. 1997; Schrufer et al. 2010). Our present result was revealed that high glucose activates expression of HIF-1α and VEGF in TR-BBB cells, brain vascular endothelial cells (Fig. 2). This result agrees with previous findings in other cell lines. Therefore, we hypothesized HIF-1 may be related to reduction of taurine uptake in high glucose in TR-BBB cells. To determine the role of HIF-1 in reduced taurine uptake by high glucose, we tested the change of taurine transport in TR-BBB cells by pre-treatment of HIF-1 inducer, cobalt chloride and HIF-1 inhibitors, 2ME2, YC-1, BA. Cobalt chloride treatments reduced the expression level of TAUT in TR-BBB cells, as in treatment of 25 mM glucose (Figs. 4 and 5). HIF-1 inhibitors recovered both taurine uptake (Fig. 3) and TAUT expression (Figs. 4 and 5) reduced by high glucose. The results verify that HIF-1 played a role in regulation of taurine transport by reducing of TAUT expression in the brain endothelial cells. Bisphosphonate drug, ALD is also used extensively to reduce circulating VEGF thereby reducing metastasis for many forms of cancer (Santini et al. 2002). Pre-treatment of ALD significantly induced [^{3}H]taurine uptake reduced by high glucose in TR-iBRB cells (Lee and Kang 2013). As in previous our result in TR-iBRB cells, ALD recovered both taurine uptake (Fig. 3) and TAUT expression (Fig. 4) in TR-BBB cells. Nonetheless, we could not conclude that HIF-1 is the sole inducer of the VEGF expression. Other factors may also be responsible for the VEGF expression, such as protein kinase C (Poulaki et al. 2002) and peroxisome proliferator-activated receptor γ cofactor-1α (O'Hagan et al. 2009). Further studies about regulation of VEGF by HIF-1 and other factors are needed.

5 Conclusion

In conclusion, taurine transport through the BBB can be down-regulated by high glucose, which is mediated to induction of HIF-1 and VEGF. Therefore, we suggest that the inhibitors of HIF-1 and VEGF could have the beneficial effects on hyperglycemia by up-regulation of taurine contents in brain.

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Thiotaurine Protects Mouse Cerebellar Granule Neurons from Potassium Deprivation-Induced Apoptosis by Inhibiting the Activation of Caspase-3

Jessica Dragotto, Elisabetta Capuozzo, Mario Fontana, Armando Curci, Maria Teresa Fiorenza, and Sonia Canterini

Abbreviations

Ara-C	Cytosine-α-D arabinofuranoside
CGNs	Cerebellar granule neurons
cyt-c	Cytochrome complex
DIV	Days *in vitro*
PI	Propidium iodide
TAU	Taurine
TTAU	Thiotaurine

1 Introduction

Death by apoptosis is a normal phenomenon in animal development, essential to eliminate supernumerary, misplaced or damaged cells with high specificity (Meier et al. 2000).

It is known that taurine (TAU), an endogenous amino acid found at high concentrations in the brain, is a neuroprotective molecule acting as osmoregulator, modulating ionic movements, regulating intracellular level of free calcium and increasing mitochondrial buffering of these ions (Kumari et al. 2013). In particular, TAU protects neurons against glutamate-induced neurotoxicity by modulating glutamate-

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induced membrane depolarization, elevation of $[Ca^{2+}]_i$, activation of calpain, reduction of Bcl-2 and apoptosis (Wu et al. 2009). Moreover, TAU prevents the amyloid-β peptide neurotoxicity in chick cultured retinal neurons by binding to $GABA_A$ receptors and increasing Cl^- conductance (Louzada et al. 2004). Several studies have recently reported novel protective effects of TAU against ischemic stroke (Menzie et al. 2013), age-related impairment of the GABAergic system (El Idrissi et al. 2013) and ethanol-induced apoptosis in the mouse cerebellum during postnatal life (Taranukhin et al. 2010).

Hypotaurine, an intermediate in the biosynthesis of TAU, reacts with sulfide to produce thiotaurine (TTAU), a thiosulfonate. Compared to TAU, the anti-apoptotic effect of TTAU has been poorly investigated so far. For instance, it is only know that addition of 100 μM TTAU to incubation medium determines a 55 % inhibition of Caspase-3 activity during the spontaneous apoptosis of human leukocytes (Capuozzo et al. 2013).

We have expanded present knowledge in the field by comparing the neuroprotective efficacy of TTAU and TAU in a well-established experimental system for studying cell survival and apoptosis, such as isolated cerebellar granule neurons (CGNs).

Since CGNs represent the largest homogeneous neuronal population of mammalian brain and are mostly generated postnatally, *in vitro* cultures of these cells can be easily obtained. Most CGNs die after 6 days in culture unless they are maintained under a chronic depolarizing condition that is obtained in a medium containing a high potassium concentration (25 mM K^+). The 25 mM K^+ condition mimics synaptic excitatory inputs these cells receive from mossy fibers *in vivo*. When CGNs are cultured in 25 mM K^+ for 6 days and shifted to a medium containing a lower potassium concentration (5 mM K^+) they undergo apoptosis within 30 min (Canterini et al. 2009) and die during the following 24–48 h, due to the hyperpolarization of plasma membrane. On the other hand, as originally performed by de Luca et al. (1996), CGNs continuously cultured in 5 mM K^+-containing medium undergo spontaneous apoptosis and show progressive accumulation of DNA breaks, chromatin condensation and nuclear fragmentation.

Besides confirming the ability of TAU to prevent neuronal death of CGNs cultured in 5 mM K^+-containing medium, our results enlighten a novel and more remarkable anti-apoptotic effect of TTAU in CGNs committed to apoptosis in the acute paradigm. In conclusion, TTAU may represent a novel class of TAU derivatives playing a key role in counteracting neuronal apoptosis.

2 Methods

2.1 Chemicals

TTAU was prepared from hypotaurine and elemental sulfur and their purity confirmed by HPLC as previously described (Capuozzo et al. 2013).

TAU was purchased from Fluka BioChemica (Sigma Aldrich, St. Louis, MO). *In vitro* culture media and related reagents were purchased from InVitrogen GIBCO (Invitrogen/GIBCO, Cralsbad, California). Chemicals were from Sigma Aldrich, unless otherwise specified.

2.2 Animals

CD1 mice of the Swiss-Webster strain were purchased from Charles River Italia (Calco, Italy) and raised in our colony. Pups were killed by decapitation without anaesthesia. Experimental protocols and procedures were approved by the Italian Ministry of Public Health and animals were raised in accordance with Sapienza University guidelines for the care and use of laboratory animals. All efforts were made to reduce the number of animals used.

2.3 **In Vitro** *Cultures of CGNs*

In vitro cultures of isolated CGNs were prepared from cerebella of PN5–6-day-old mice as previously described (Canterini et al. 2012). Briefly, cerebella were rapidly dissected from the brain, minced into small pieces, incubated at RT for 15 min in digestion buffer (containing 0.1 % trypsin and 100 μg/mL DNase in PBS) and repeatedly passed through a flame-polished glass pipette until a single-cell suspension was obtained. Cells were then recovered by centrifugation and suspended in DMEM culture medium containing 25 mM K^+, 2 mM glutamine, 2 % B27 (Gibco), 100 units/mL penicillin and 100 μg/mL streptomycin, and 5 % fetal bovine serum (FBS), then plated at a density of 1×10^5 cells/well culture multiwell (previously coated with 0.1 μg/mL poly-L-lysine). CGNs were either continuously cultured for 6 days in a medium containing a standard potassium concentration (5 mM K^+) in the presence/absence of 1 mM TAU or TTAU (chronic paradigm) or in a medium containing an high concentration of potassium (25 mM K^+). Under the 25 mM K^+ condition, CGNs differentiate and increase neurite sprouting and elongation. Cell shift from 25 to 5 mM K^+ and treatment with 1 mM of TAU or TTAU for 16 h were performed after 6 days of *in vitro* culture (DIV6) and were preceded by a 12 h incubation in serum-free medium (acute paradigm).

Routinely, glial cell proliferation was inhibited by supplementing the culture medium with 10 μM cytosine-α-D arabinofuranoside (Ara-C; Sigma Aldrich) 18–22 h after plating.

2.4 Detection of Cellular Viability

In vitro cell viability was estimated by staining nuclei with Hoechst 33258 (Sigma Aldrich) and propidium iodide (PI) (Sigma Aldrich). Because plasma membranes of live cells are not permeant to PI, it was used to detect dead cells, whereas Hoechst staining allowed to count the total number of nuclei (Iyer et al. 1998).

Following treatments, cells were incubated with Hoechst 33258 (10 μM) and PI (10 μM) for 15 min at 37 °C and observed under an epifluorescence microscope (Leica Microsystem, Milan, Italy) to determine the number of Hoechst 33258- and PI-positive cells, respectively. The number of cells counted was approximately 1,000 cells for each independent observation (3 randomly acquired microscopic fields). Images were acquired and analyzed using the Metamorph 5.5 software.

2.5 Detection of Caspase-3 Protein Expression by Western Blotting Assays

To analyze the level of Caspase-3, CGNs were quickly rinsed with chilled PBS, detached/collected using a plastic scraper and homogenized in RIPA buffer (Sigma Aldrich) supplemented with protease-phosphatase inhibitors (Protease and Phosphatase Inhibitor Cocktails, Roche Diagnostics, Milan, Italy). Homogenates were centrifuged at 10,000 × *g* for 15 min at 4 °C and supernatants were analysed by Western Blotting (WB). Protein concentration was routinely determined by DC Protein Assay (Bio-Rad, Milan, Italy) loading equal amounts of total protein/lane on 4–20 % gradient Mini-Protean TGX precast gel for electrophoresis (Bio-Rad, Milan, Italy). Fractionated proteins were transferred to PVDF membranes (Roche, Milan, Italy) and then processed for WB using an anti-Caspase-3 rabbit polyclonal (Sigma Aldrich; 1:400 dilution), anti-cleaved Caspase-3 rabbit polyclonal (Cell Signaling Technology, Danvas, MA, USA; 1:600 dilution), and anti-β III Tubulin mouse polyclonal (AbCam, Cambridge, UK; 1:400 dilution) antibodies. Secondary antibodies used were anti-rabbit HRP-conjugated goat polyclonal (Pierce, Rockford, IL, USA; 1:650 dilution) and anti-mouse HRP-conjugated horse polyclonal (Pierce; 1:650 dilution) antibodies. Chemiluminescent protein bands were revealed using SuperSignal West Dura reagents (Pierce). Caspase-3 protein was normalized to β III Tubulin levels.

2.6 Statistics

In all figures, histograms represent the mean ± SEM of data obtained in three independent experiments and having at least three replicates for each data point. Graphics and statistical significance was determined using SPSS software.

3 Results

3.1 *TAU and TTAU Likewise Rescue the Death of CGNs Cultured in 5 mM K^+-Containing Medium (Chronic Paradigm)*

We have first investigated the neuroprotective effect of TAU and TTAU on CGNs continuously cultured in 5 mM K^+ up to 6 days (chronic paradigm).

To this end, CGNs were cultured for 6 days either in the absence (5 mM K^+) or in the presence of 1 mM of TAU (5 mM K^+ + TAU) or 1 mM of TTAU (5 mM K^+ + TTAU). In parallel, CGNs were also continuously cultured in high potassium-containing medium (25 mM K^+).

Cell viability was then assessed by determining the number of apoptotic nuclei after staining unfixed cells with Hoechst (to stain all nuclei) and PI dye (to stain dead cells). The fraction of dead cells was then determined by visualization under an epifluorescence microscope.

Figure 1 shows that CGNs cultured in 25 mM K^+ were mostly alive with only a small fraction (8 %) of PI-positive cells, whereas CGN cultures maintained in 5 mM K^+ displayed a 70 % of dead cells. The presence of 1 mM either TAU (5 mM K^+ + TAU) or TTAU (5 mM K^+ + TTAU) reduced the fraction of dead cells to 20 %. Together, these data indicate that TTAU is a potent protective molecule in this apoptotic system having an efficacy similar to that of TAU, a well-established neuroprotector (Louzada et al. 2004).

3.2 *Compared to TAU, TTAU Rescues More Efficiently CGNs from Potassium Deprivation-Induced Apoptosis (Acute Paradigm)*

To gain more insights on the mechanisms underlying TAU and TTAU efficacy in counteracting apoptotic death, we next exploited the acute paradigm to induce apoptosis of CGNs. Since, as mentioned in the introduction, in this paradigm CGNs are triggered to apoptosis after they are fully differentiated, these experiments were aimed at investigating the rescuing ability of TAU and TTAU in a condition mimicking the physiological context in which these cells usually are. To this end, DIV6 CGNs continuously maintained in 25 mM K^+ were triggered to apoptosis by lowering potassium in the culture medium in the absence of TAU or TTAU (5 mM K^+) or in the presence of 1 mM either TAU (5 mM K^+ + TAU) or TTAU (5 mM K^+ + TTAU). After 24 h cell viability was assessed by Hoechst and PI staining, as described in the previous paragraph. In parallel, CGNs were also continuously cultured in high potassium-containing medium (25 mM K^+).

Exposure to 5 mM K^+ resulted in approximately 70 % cell death (Fig. 2), whereas 5 mM K^+ medium supplementation with TAU partially protected CGNs from death,

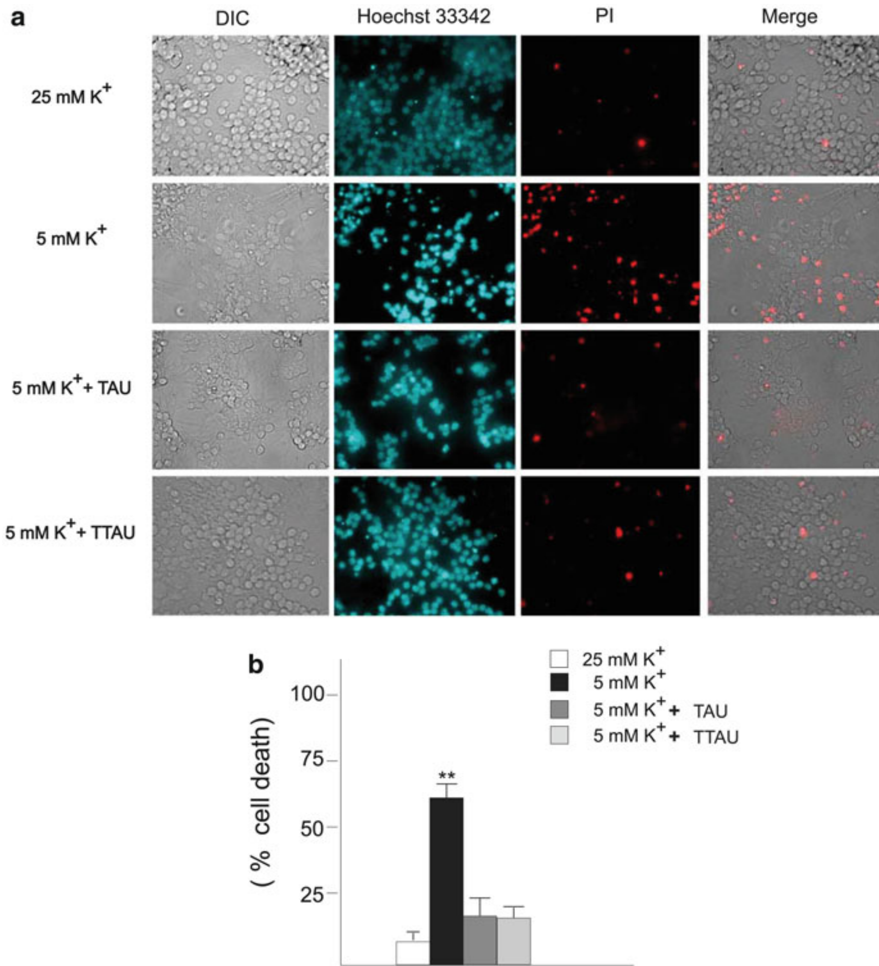

Fig. 1 (**a**) Hoechst (nuclei) and propidium iodide (PI, apoptotic nuclei) double staining of CGNs that were cultured in $K5^+$-containing medium for 6 days (chronic paradigm) either in the absence (5 mM K^+) or in the presence of 1 mM of TAU (5 mM K^++TAU) or 1 mM of TTAU (5 mM K^++TTAU). Scale bar indicates 30 μm. (**b**) Histograms represent the fraction of dead cells, determined by PI staining (*red*)

reducing the fraction of death cells to 35 % of total cells. Interestingly, the supplementation of 5 mM K^+ medium with TTAU more efficiently improved cell survival further reducing the fraction of dead cells to 10 % of total cells.

Given that molecular events downstream from potassium dyshomeostasis in CGNs include the activation of Caspase-3 by proteolysis of the pro Caspase-3 (Mora et al. 2001), we decided to evaluate whether TTAU prevented the proteolytic cleavage of this pro-enzyme.

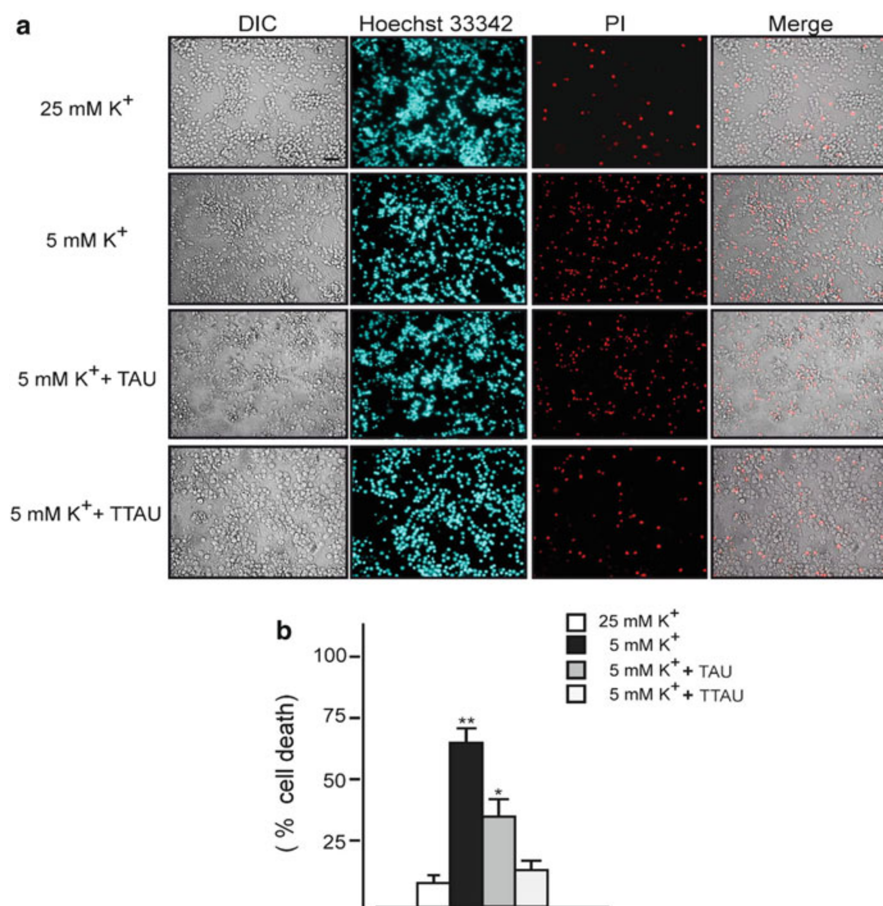

Fig. 2 (**a**) Hoechst (nuclei) and propidium iodide (PI, apoptotic nuclei) double staining of CGNs shifted to 5 mM K^+ medium for 24 h (acute paradigm) after 6 days of culture in 25 mM K^+ medium (depolarizing, pro-survival condition) either alone (5 mM K^+, apoptotic condition) or in the presence of 1 mM TAU (5 mM K^++TAU) or 1 mM TTAU (5 mM K^++TTAU). Scale bar indicates 30 μm. (**b**) Histogram represents the fraction of dead cells, detected using propidium iodide (*red* staining)

To this end, we determined the amount of activated Caspase-3 by performing WB assays of total proteins extracted from CGNs 16 h after the shifting to 5 mM K^+ medium. Both the uncleaved and the cleaved form of Caspase-3 were detected using specific antibodies and their relative abundance was expressed as ratio between cleaved Caspase-3 and β III Tubulin standard.

While the expression levels of pro Caspase-3 were not changed under the various conditions, K^+ deprivation was marked by a sevenfold increase of activated Caspase-3 compared to the 25 mM K^+ condition. The supplementation of 5 mM K^+ medium with TAU or TTAU significantly reduced and precluded Caspase-3 activation, respectively (Fig. 3).

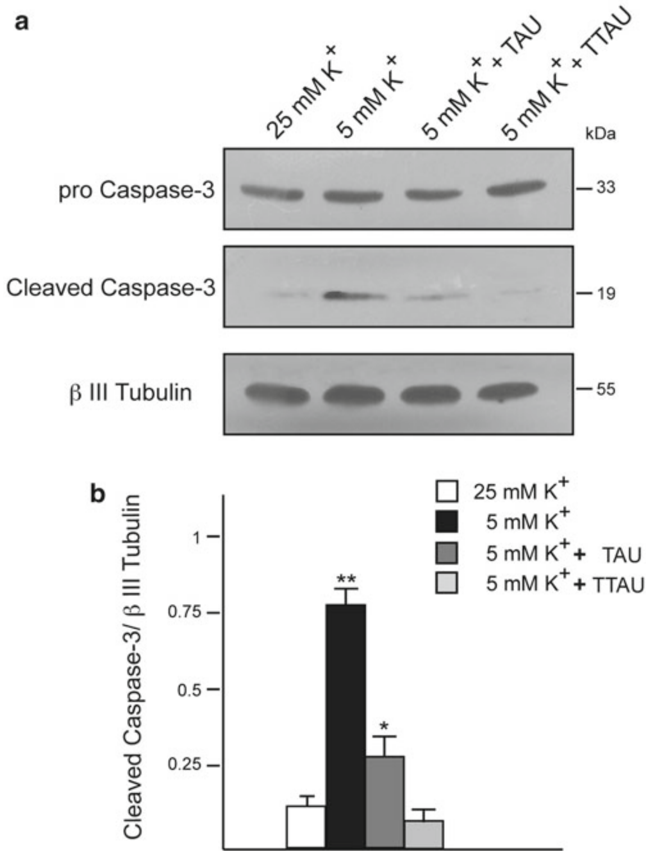

Fig. 3 (**a**) Total protein extracts of CGNs in acute paradigm were processed for WB with anti-pro Caspase-3 and anti-cleaved Caspase-3. (**b**) The relative abundance of cleaved Caspase-3 protein bands was expressed as ratio between pixels of cleaved Caspase-3 and β III Tubulin bands. Histograms represent the mean ± SEM of the ratios obtained in three independent experiments

Taken together these results strongly suggest that when apoptosis is induced by potassium-deprivation in the acute paradigm the TTAU directly prevents the activation of Caspase-3.

4 Discussion

This study provides novel inside on the anti-apoptotic activities of TAU and TTAU in a well-established model system of neuronal apoptosis (Gallo et al. 1987).

The chronic and acute paradigms exploited in this study allowed us to investigate the neuroprotective effects of TAU and TTAU in neuronal cells that markedly differ

in terms of differentiative traits and spontaneously die or can be triggered to die, respectively. In fact, CGNs that are cultured in 5 mM K^+-containing medium (chronic paradigm) are functionally immature and spontaneously die after DIV6 entering a death program that is not mediated by cytochrome complex (cyt-c) release from mitochondria and Caspase-9 activation (Alavez et al. 2003). By contrast, CGNs that are cultured in 25 mM K^+-containing medium for more than 5–6 days fully differentiate and can be committed to apoptosis by the exposure to a 5 mM K^+-containing medium (acute paradigm). Thereafter, they die within 24–48 h, showing typical feature of apoptotic death, including DNA condensation and Caspase-3 activation (Moran et al. 1999).

The responsiveness of CGNs to K^+ deprivation is acquired during *in vitro* differentiation; DIV3-4 CGNs do not respond to the shift in 5 mM K^+ in terms of commitment to apoptosis because they have not yet developed the dependence on membrane electrical activity for survival (de Luca et al. 1996).

Our findings indicate that under the chronic paradigm TAU and TTAU share a similar efficacy in rescuing the survival of CGNs, indicating that TAU and TTAU promote CGN survival through a similar pathway(s). However, in spite of the wealth of information available on the neuroprotective effect of TAU, the role of TTAU has been poorly investigated so far. Among the pathways responsible for the pro-survival effect of TAU in our chronic paradigm, we favor the hypothesis that a regulation of the homeostasis of intracellular K^+ concentration plays a major role. In fact, it has been reported that TAU inhibits several classes of K^+ channels, including Ca^{2+}-activated K^+ channels (Tricarico et al. 2001) and influences K^+ conductance through the inhibition of inward voltage dependent K^+ channels (Kv) (Bulley et al. 2013).

Under the acute paradigm, TTAU more efficiently improves cell survival compared to the very weak effect of TAU. This difference likely relies on the specific feature of the apoptosis induced by K^+-deprivation in fully differentiated CGNs, as outlined above. Apoptosis triggered by the acute paradigm is marked by an early-phase (0–3 h after the apoptotic stimulus) in which ROS production increases and cyt-c release from mitochondria occurs to begin Caspase-3 activation. Then, during a later phase (3–15 h after the apoptotic stimulus) proteosomes activity decreases, Caspase-3 activity increases, cyt-c is degraded and ROS levels remain still high (Atlante et al. 2003).

Because TTAU is a potent anti-oxidant (Acharya and Lau-Cam 2013) and a biochemical intermediate in the transport, storage, and release of sulfide (Pruski and Fiala-Médioni 2003; Capuozzo et al. 2013), we believe that its strong efficacy in counteracting apoptosis in the acute paradigm mostly relies on the ability to reduce the level of ROS. However, Capuozzo et al. (2013) suggested that TTAU prevents apoptosis of human neutrophils by generating H_2S from sulfane sulfur atom, a gaseous molecule that has a regulatory activity on inflammatory responses (Zanardo et al. 2006). Moreover, it is widely recognized that H_2S promotes the short-term survival of neutrophils by inhibiting of Caspase-3 cleavage (Rinaldi et al. 2006) and exerts its anti-aging effects by directly increasing the inhibitory effects of GSH and SOD on ROS production and the redox enzyme levels improving the resistance of

cell to stress (Zhang et al. 2013). In spite of the different efficacy displayed by TAU and TTAU in the acute paradigm, results of Fig. 3 indicate that their pro-survival effect is dependent on the ability to inhibit Caspase-3 activation, indicating a specific role in the early phase of cell commitment to apoptosis.

5 Conclusion

Our study adds novel inside on the anti-apoptotic activity of TTAU, the biological relevance of which is still a challenge to biochemical research, by showing that it strongly inhibits Caspase-3 activation. This pinpoints an important role of this molecule in the biochemical changes associated with early phases of apoptosis in differentiated and functionally mature neurons.

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Experimental Study of Taurine on the Cerebral Dehydrogenase Activity in the Model of Unilateral Cerebral Ischemia

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1 Introduction

Ischemic brain affection is a common outcome of different diseases: diabetes mellitus, arterial hypertension, coronary artery disease, etc. (Tanashyan 2007; Suslina et al. 2009). They also could be caused by previously done coronary artery bypass graft (Shrader et al. 2012) and surgeries on the common carotid arteries (Ponomarev et al. 2011). According to statistics, 5–10 % of all human strokes are due to atherosclerotic thrombosis of the left common carotid artery (LCCA) (Mollaev et al. 2013). It is reported that there is significant morphological, biochemical and functional difference between brain hemispheres (Bogolepov and Fokin 2004). If one of the hemispheres is deteriorated during brain ischemia, the functional deficiency will be balanced by the symmetrical structures of the other hemisphere (Smusin et al. 2001).

Brain ischemia could lead to unstable connections between both hemispheres (Fokin 2012). More recently, it has been shown that, after a stroke in the LCCA circulation, the intact hemisphere was also deteriorated in a significantly greater number of patients (Makarova and Pogoreliy 2010). Moreover, severe stroke in the LCCA circulation leads to severer deteriorations in the central nervous system and

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causes more physical disabilities than stroke in the right common carotid artery (RCCA) circulation (Smusin et al. 2001).

Taurine is known to be of vital importance in the central neural system functioning. Taking into consideration taurine's neuroprotective activity it seems advisable to do an experimental study of taurine after unilateral occlusion of the left or right common carotid artery.

2 Methods

2.1 Experimental Materials and Methods

To model acute brain ischemia unilateral occlusion of the common carotid artery (left or right) was performed. In Fig. 1 ptosis on the left eye is clearly seen as an indicator for brain tissue deterioration after occlusion of the left common carotid artery.

After unilateral occlusion of the common carotid artery (left or right) animals were treated with intraperitoneal administration of taurine 100 mg/kg once daily during 3 days. After 72 h of experiment animals were decapitated under chloral hydrate general anesthesia (300 mg/kg) and brains were extracted. Brains were rapidly bathed in the normal saline; hemispheres were split along the midline and were placed in the freezer under −18 °C. Further frozen hemispheres were weighed, divided into 6–8 equal parts and placed into a glass weighing cup.

Phosphate buffer containing triphenyl tetrazolium chloride (100 mg per 100 ml) was added to the tissues at +37 °C at a 1:9 ratio (brain tissue: buffer). After that samples in the containers were placed into a temperature controlled environment and maintained for 90 min at +37 °C. Triphenyl tetrazolium chloride (TPH) was used as a damage zone indicator. Brain tissue with preserved dehydrogenase activity undergoes a reduction reaction with TPH reduced to formazan (Figs. 2 and 3).

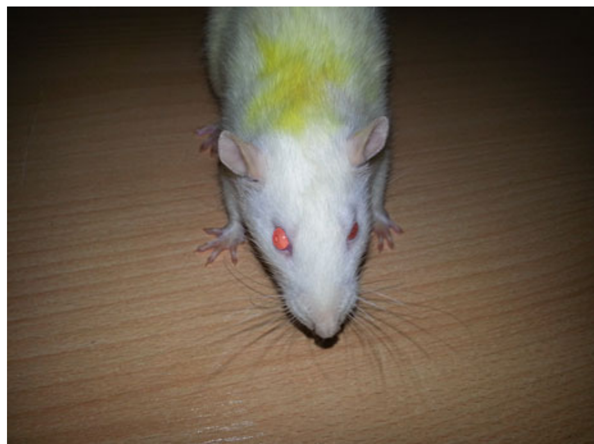

Fig. 1 Ptosis on the left eye is clearly seen as an indicator for brain tissue deterioration after occlusion of the left common carotid artery

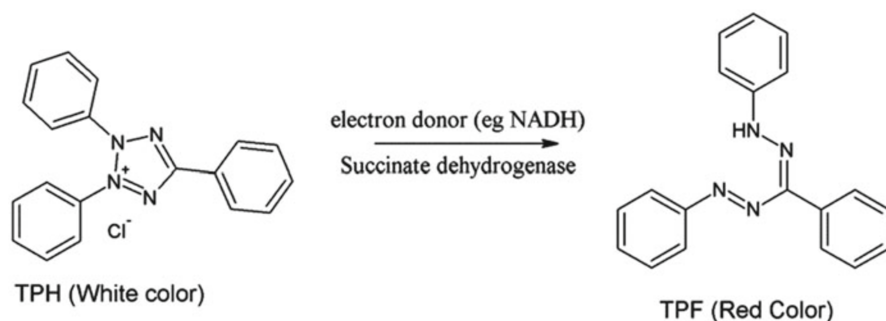

Fig. 2 Reduction of triphenyl tetrazolium chloride to formazan

Fig. 3 Brain tissue with preserved dehydrogenase activity (*red* color) undergoes a reduction reaction with TPH that reduced to formazan

Brain tissue viability was investigated 24 h after reaction. Formazan chloroform extraction was performed (5 ml of chloroform per 1 ml of tissue homogenate). Extraction was done by shaking the tissue homogenate for 30 s every 5 min for 20 min at +4 °C. After that, the homogenate was centrifuged for 5 min at 1,500 (±150) × g to separate chloroform. Formazan concentration was measured twice by spectrophotometric determination ($\lambda = 490$ nm) on spectrophotometer SF-46: immediately after reaction and 24 h holding after reaction. Formazan concentration (μg) was indicated by calibration schedule for 1 g of brain tissue.

Hemispheric asymmetry ratio (Ka) of the right hemisphere compared to the left hemisphere was calculated using a formula:

$$Ka = (S - D) / (S + D) \times 100$$

Where S Formazan amount in the left hemisphere, D Formazan amount in the right hemisphere.

Asymmetric shift in the ischemic hemisphere compared to the intact hemisphere was counted as "% of asymmetric shift."

2.2 Statistic Analysis

Statistical significance was determined by Student's t-test. Each value was expressed as the mean ± SEM. Differences were considered statistically significant when the calculated P value was less than 0.05.

3 Results

It was found out that after occlusion of the left common carotid artery, taurine blocked formazan quantity decrease compared to control group (Table 1). The percent of formazan quantity increase was 94.6 % in the damaged hemisphere and 53.7 % in the healthy hemisphere compared to control group. After occlusion of the right common carotid artery taurine increased formazan quantity formation to 60.3 % in the damaged and to 29.1 % in the healthy hemisphere. After occlusion of the right common carotid artery brain tissue viability rate was +2.9 % in the left hemisphere and +4.8 % in the right hemisphere compared to +1.6 % and +2.6 % respectively in the control group. After occlusion of the left common carotid artery brain tissue viability rate was +2.8 % in the left hemisphere and +6.0 % in the right hemisphere compared to +1.0 % and +2.3 % respectively in the control group.

Table 1 Formazan concentration (μg/kg) in the Taurine group immediately after reaction (A) and 24 h holding after reaction (B) in the models of LCCA and RCCA occlusion

Experimental groups	Experimental conditions		Formazan concentration (μg/kg)		% of asymmetric shift	Ka
			Left hemisphere	Right hemisphere		
Sham operated animals	No occlusion	A	375.5 ± 20.0	399.5 ± 26.0	−6.7 ± 4.0	−3.1 ± 1.6
		B	415.5 ± 18.5	423.0 ± 17.5	−3.7 ± 5.0	−0.9 ± 1.5
Control	LCCA occlusion	A	165.4 ± 4.1$^{\#}$	254.2 ± 2.3	−34.9 ± 1.6$^{\#,}$&	−14.1 ± 1.9&
		B	237.1 ± 5.1$^{\#}$	303.8 ± 6.0	−15.5 ± 3.3$^{\#,}$&	−14.6 ± 1.6&
	RCCA occlusion	A	251.4 ± 5.2	250.2 ± 4.9	−0.2 ± 2.3	−1.7 ± 2.8
		B	294.5 ± 4.6	294.8 ± 4.3$^{\#}$	0.25 ± 1.9$^{\#,}$&	−7.5 ± 1.8&
Taurine	LCCA occlusion	A	322.0 ± 10.0*	391.0 ± 16.0*	−16.0 ± 4.8&,*	−9.7 ± 0.6$^{\#,*}$
		B	425.5 ± 10.0*	487.0 ± 10.5$^{\#,*}$	−6.7 ± 1.2&,*	−6.7 ± 0.2$^{\#,}$&,*
	RCCA occlusion	A	333.5 ± 19.0	401.5 ± 15.0	−23.7 ± 5.6&,*	−9.3 ± 0.9$^{\#,}$&,*
		B	460.0 ± 24.0	516.5 ± 28.5$^{\#}$	+13.5 ± 4.7&,*	−5.8 ± 0.2$^{\#,}$&,*

$p < 0.05$ compared to:
*Control group
[#]Sham operated animals
&Significant values of % of asymmetric shift
A—Formazan concentration (mg/kg) immediately after reaction
B—Formazan concentration (mg/kg) 24 h holding after reaction

4 Discussion

Effectiveness of neuroprotective drugs during unilateral occlusion of the common carotid artery is understudied problem. Though it is widely known that 5–10 % of all human strokes are due to atherosclerotic thrombosis of the left common carotid artery (Mollaev et al. 2013). For this reason in our experiments we investigated taurine influence on the ischemic brain tissue viability of the right and left hemisphere, ischemic damage area after unilateral occlusion of the common carotid artery (left or right).

It was found out that taurine could preserve cerebral metabolism after unilateral ischemia (compared to control group). Though we'd like to admit that therapy effectiveness depended on ischemic zone localization. For example taurine was very effective during occlusion of the LCCA and it was active in the deteriorated hemisphere.

During occlusion of the RCCA we have got other results. Taurine was not as active during this pathology. Experiment demonstrated positive impact of taurine on the cerebral metabolism 24 h after occlusion in the RCCA.

Therefore, in our experimental work we have demonstrated taurine activity during cerebral ischema. Significant impact of taurine on the main pathogenetic factors of ischemic and reperfusion injury are explained by the role taurine in the central neural system (as neurotransmitter). Taurine's effects in the cerebral tissue could be related to it's metabolic activity, antioxidant activity, positive influence on the carbohydrate's metabolism, decreasing of lipid peroxidation, succinate dehydrogenase preservation, maintaining the structural integrity of the membrane, maintaining the autoregulation phenomenon, limiting the postischemic phenomenon, decreasing of hypercoagulation (Oleynikova et al. 2009; Makarova et al. 2014). We'd like to admit that taurine could be used not only for prophylaxis, but for therapeutic treatment of cerebral diseases.

5 Conclusion

Experimental study has demonstrated that therapeutic administration of taurine in the dose of 100 mg/kg has limited dehydrogenase activity dysfunction after occlusion of the left common carotid artery either in the deteriorated and intact hemispheres.

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Taurine Targets the GluN2b-Containing NMDA Receptor Subtype

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Abbreviations

DL-AP5	DL-2-amino-5-phosphonovalerate
GABA	γ-Aminobutyric acid
IC_{50}	Half maximal inhibitory concentration
NMDA	*N*-methyl-D-aspartic acid
TAG	6-Aminomethyl-3-methyl-4H-1,2,4-benzothiadiazine 1,1-dioxide
TBST	Tween 20-containing tris-buffered saline

1 Introduction

Taurine (2-amino-ethanesulfonic acid) is abundantly found in mammalian excitable tissues and appears to serve as an inhibitory neuromodulator in the CNS. Supportive evidence for this role includes its association with synaptic membrane structures (Kontro and Oja 1987) and its presence and co-localization with its synthesizing enzyme, cysteine sulfonic acid decarboxylase, in pre-synaptic neuronal terminals (Wu et al. 1979; Wu 1982; Magnusson et al. 1989). Stimulated taurine release

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(Philibert et al. 1989) as well as the sodium-dependent taurine transporter (Kozlowski et al. 2008) has also been demonstrated. There is also strong evidence for the existence of a specific taurine receptor (Yarbrough et al. 1981; Okamoto et al. 1983a, b; Frosini et al. 2003a, b; Martin et al. 1981). Taurine is involved in numerous physiological functions and its moderate inhibitory neuromodulatory role makes it a superior candidate as a neuroprotective agent (Chen et al. 2001).

It is well established that excitotoxicity results from excessive activation of metabotropic and ionotropic glutamate receptors (Coyle and Puttfarcken 1993; Ikonomidou et al. 1999; Besancon et al. 2008). Previous investigators have demonstrated that taurine may protect neurons from excitotoxicity either by decreasing the intracellular levels of free Ca^{2+}, or by opposing the actions of glutamate (El Idrissi and Trenkner 1999; Chen et al. 2001; Saransaari and Oja 2000; Lidsky et al. 1995). Several mechanisms may explain the ability of taurine to prevent the glutamate-induced rise in cytosolic Ca^{2+}. These include blockade of glutamate-activated Ca^{2+} influx via reverse-mode operation of the Na^+/Ca^{2+} exchanger and inhibition of Ca^{2+} release from intracellular storage (El Idrissi and Trenkner 1999, 2003). Taurine was shown to inhibit L-, N-, and P/Q- voltage-gated calcium channels (Wu et al. 2005; Liu et al. 2006). Another mechanism by which taurine opposes the actions of glutamate, albeit indirect, involves the opening of Cl^- channels by taurine demonstrated in various neuronal preparations (Yarbrough et al. 1981; Oja et al. 1990; Belluzzi et al. 2004). This is likely mediated through activation of a specific receptor that is distinct from the GABA (γ-aminobutyric acid)-A or -B receptors or the strychnine-sensitive glycine receptor. Activation of this Cl^--channel gating receptor is inhibited by the synthetic ligand, 6-aminomethyl-3-methyl-4H-1,2,4-benzothiadiazine 1,1-dioxide (TAG, Yarbrough et al. 1981; Frosini et al. 2003a, b; Martin et al. 1981) and may indeed be a major mechanism by which taurine limits glutamate actions. We refer to this site as the TAG-sensitive taurine receptor.

Nonetheless, it remains unclear whether taurine may additionally act through other sites or mechanisms to directly modulate glutamate NMDA or AMPA receptor subtypes. In our laboratories, we tested these possibilities by adopting electrophysiological, receptor binding and western blotting techniques. The data suggest that taurine selectively diminishes responses mediated by the NMDA-, but not the AMPA-glutamate receptor subtype, as this inhibitory action of taurine was prevented by pretreatment of slices with a near-saturating concentration of the specific inhibitors of the NMDA receptor, DL-AP5 or MK801, (Chan et al. 2013, 2014). The data furthermore demonstrate a picrotoxin-insensitivity: Since the TAG-sensitive receptor is non-competitively blocked by picrotoxin, this points to the existence of a TAG-insensitive taurine action on glutamatergic transmission. This action is also distinct from one that is mediated through blocking of a L-type Ca^{2+} channel (Wu et al. 2005), as it was not prevented by nifedipine (Chan et al. 2014).

Direct interaction between taurine and the NMDA receptor was also evident in receptor binding studies. In these experiments, taurine was shown to dose-dependently antagonize the spermine-enhanced glycine-induced binding of [^{3}H] MK-801, suggesting that taurine competes with spermine for the polyamine site on the NMDA receptor (Chan et al. 2013, 2014). Without spermine, taurine had no effect on specific binding of [^{3}H] MK-801 to rat cortical membranes in the presence

of either glutamate or glycine. These results indicate that taurine interacts with a polyamine site on the NMDA receptor, and since taurine's ability to inhibit spermine-enhanced [^{3}H] MK-801 binding to NMDA receptor is not altered by TAG, we have identified this site as the TAG-insensitive taurine receptor (Chan et al. 2014).

This mode of interaction of taurine with the NMDA receptor finds a parallel in an earlier study of acamprosate, a synthetic analog of taurine. Acomprosate also failed to alter NMDA- or glutamate-induced currents in primary cultured striatal and cerebellar granule cells; but 10 μM acamprosate diminished the spermine-mediated enhancement of the NMDA-induced peak current by 10 %, sparing the steady-state current in a sub-population of striatal neurons (Popp and Lovinger 2000). Thus, taurine and acamprosate appear to interact with the polyamine site in the NMDA receptor to partially inhibit spermine actions. Multiple effects of spermine on the NMDA receptor have been described (Williams 1997a, b). Moreover, the apparent affinity of the NMDA receptor for glycine in the presence of 0.1 mM spermine was found to be reduced tenfold by 0.1 mM taurine (Chan et al. 2014). Thus, electrophysiological and receptor binding data strongly implicate a specific interaction between taurine and NMDA receptor at or adjacent to the polyamine site.

Functional NMDA receptors comprise multiple subunits including GluN1, GluN2 A, B, C, and D, as well as GluN3 A, and B, (Mori and Mishina 1995; Dingledine et al. 1999). The best-understood form of the receptors are tetramers containing two GluN1 and two GluN2 (or two GluN3), with the specific constituent of the GluN2 (or GluN3) group determining electrophysiological properties and drug binding profile of the NMDA receptor subtypes (Burnashev et al. 1995). Polyamines exhibit multiple effects on NMDA receptors, including enhancement of NMDA-receptor gated currents in the presence of saturating concentrations of glycine, an increase and decrease in the apparent affinities of NMDA receptor for glycine and glutamate respectively, and voltage-dependent inhibition when applied extracellularly (Williams 1997a). Interestingly, while the GluN1A/GluN2B subtype exhibits all 4 effects, the GluN1A/GluN2A subtype shows only an increase in the apparent agonist affinity and the voltage-dependent form of inhibition by polyamines. GluN1A/GluN2C or 2D subtypes do not interact with polyamines (Williams 1997b). Both [^{14}C]spermine binding and functional assays show a several-fold preference of spermine for the terminal regulatory domain of the GluN2B to that of the GluN2A (Han et al. 2008; Masuko et al. 1999; Mutel et al. 1998; Fischer et al. 1997).

In the present study we seek to identify the specific NMDA receptor subtype that interacts with taurine. Our first approach was to investigate the effect of Ro25-6981, a known non-competitive antagonist of GluN2B-containing NMDA receptor, on the inhibitory modulatory action of taurine on evoked responses in the rat brain slice. We also studied the ability of polyamines to compete with taurine binding in rat frontal cortex, and, using western blotting, the effects of chronic taurine treatment on membrane expression of subunits of the NMDA and AMPA receptors. The results indicate that taurine exerts a modulatory interaction with the GluN1/GluN2B receptor subtype possibly through actions on a polyamine site, and this is supported by a long-term effect on the membrane expression of GluN2B, though not the GluN1 subunit. The binding study result showed the interaction of taurine and the polyamine site(s), thus supporting the GluN2B subunit as a molecular target for taurine.

2 Methods

2.1 Slice Preparation and Field Potential Recording and Analysis

We conducted electrophysiological experiments on 400-μm rat brain prefrontal cortical slices prepared by slicing at 30° to the coronal plane, such that the dorsal edge contained the most anterior tissue in a slice, as described previously (Chan et al. 2014). Evoked field potential responses were elicited in superficial layer-5 of the prelimbic area by single pulses delivered via concentric bipolar electrodes placed medial to the nucleus accumbens that activated afferent inputs arising mostly from basolateral amygdala (Orozco-Cabal et al. 2006).

All control and drug-modulated evoked responses were acquired in the presence of 80 μM picrotoxin dissolved in artificial cerebral spinal fluid pre-equilibrated with 5 % CO_2 and 95 % O_2, and recorded with standard field potential recording techniques using a dc amplifier (Axoclamp 2A, Axon Instruments), digitized and frame averaged (Snapmastter 3.5 software, HEM Corporation). The time-course of the effect of a bath-applied drug was revealed by subtracting the evoked response in the presence of the drug(s) from the control response to yield a difference wave. The peak amplitude of the difference waveform was expressed as a percentage of the control response measured at the same time as the peak of the difference wave. Time elapsed between the stimulus artifact and the peak of a difference wave was recorded as the latency-to-peak of a drug effect.

2.2 Radioligand Binding Assay

Specific binding of [^{3}H]spermidine and [^{3}H]taurine to rat frontal cortical membrane preparations were conducted by following the procedure previously described (Chan et al. 2014).

2.3 Preparation of Synaptosomes, Fractionation and Western Blot Analysis

Synaptosomes (P2 fraction) were prepared from frontal cortices as previously described (Wang et al. 1994, 1999; Bakshi et al. 2009) with modifications. To further purify synaptosomal fractions, the synaptosome-enriched P2 fraction was washed twice in 5 ml of ice-cold Kreb's-Ringer solution enhanced with 100 μM ascorbic acid, 50 μ g/ml leupeptin, 10 μ g/ml aprotinin, 2 μ g/ml soybean trypsin inhibitor, 0.04 mM PMSF, 0.1 mM 2-mercaptoethanol, 10 mM NaF, 1 mM Na_2VO_4 and 0.5 μ l/ml protein phosphatase inhibitor I & II cocktails. To obtain cytosolic and membranous fractions of the synaptosomes, the washed synaptosomes were

sonicated for 10 s on ice in 0.5 ml hypotonic homogenization solution (25 mM HEPES, pH 7.4; 120 mM NaCl, 4.8 mM KCl, 25 mM $NaHCO_3$, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 10 mM glucose, 100 μ M ascorbic acid, 50 μ g/ml leupeptin, 10 μ g/ml aprotinin, 2 μ g/ml soybean trypsin inhibitor, 0.04 mM PMSF and 0.1 mM 2-mercaptoethanol, 10 mM NaF, 1 mM Na_2VO_4 and 0.5 μ l/ml protein phosphatase inhibitor I & II cocktails). Samples were centrifuged at 50,000 x g for 30 min. The resulting supernatant was saved as the cytosolic fraction and the synaptic membrane pellet was resuspended in 0.5 ml of hypotonic solution. Protein concentrations of the synaptic membranes were determined using the Bradford method before solubilization by adding 2× or 6× SDS-PAGE sample preparation buffer and boiled for 5 min.

Western Blotting Analysis: Proteins were size-fractionated on 7.5 % SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. The membranes were washed with TBS (Tris-buffered saline) and blocked overnight (4 °C) with 5 % milk in 0.1 % Tween 20-containing TBS (TBST). Following three 5-min washes with 0.1 % TBST, membranes were incubated at room temperature for 2 h with 1:500 to 1:1,000 dilutions of selected antibodies. After three 2-min 0.1 % TBST washes, membranes were incubated for 1 h with anti-species IgG-HRP (1:5,000–1:10,000 dilution) and washed three times with 0.1 % TBST for 2 min each. The signals were detected using a chemiluminescent method and visualized by exposure to x-ray film. Specific bands were quantified by densitometric scanning (GS-800 calibrated densitometer, Bio-Rad).

3 Results

3.1 Effects of Taurine and Ro25-6981 Had Similar Time-Courses

We tested the inhibitory action of the GluN2B antagonist, Ro25-6981 on the field potential in superficial layer-5 of medial prefrontal cortex evoked by stimulation of ventral medial cortical area in rat brain slices, and compared it with that of 2 mM taurine co-applied with Ro25-6981. In all 27 slices tested, various doses (0.2–10 μM) of Ro25-6981 clearly inhibited the late part of the N2 component, which is predominately mediated by the NMDA receptor (Chan et al. 2014). In some slices, Ro25-6981 in high doses caused an increase in the N1 and early part of N2, possibly due to disynaptic disinhibition of principle neurons, i.e. through suppression of excitation of inhibitory interneurons (Fig. 1a, b). No evidence of disinhibition was observed in latter parts of N2.

The time course of the effects of Ro25-6981 and of Ro25-6981 co-applied with taurine was revealed by subtracting the evoked response in the presence of each drug solution from the control response (Fig. 1b). These difference waveforms showed their maximum effects at a later time than the drug-free control ($p < 0.05$), reflecting that selective postsynaptic neuronal groups were responsive to each drug.

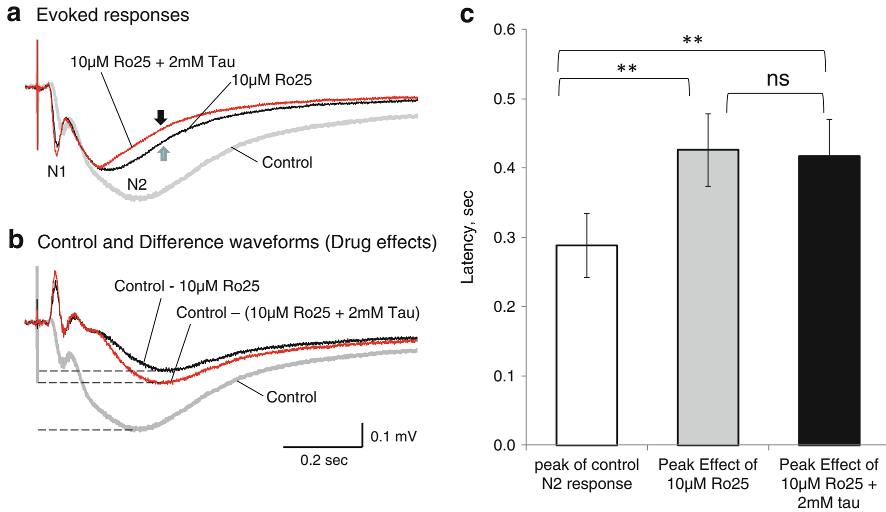

Fig. 1 Inhibition by Ro25-6981 (10 μM) and by Ro25-6981 co-applied with taurine (2 mM) are shown in panel (**a**) as selective reduction of the late part of N2 and apparent disinhibition of N1. Subtraction of these 2 responses in panel (**a**) from control yields the difference waves in panel (**b**), which represent the respective effects of the 2 drug solutions. Positive spike in (**b**) corresponding in time to N1 represents early disinhibition. Peaks of the difference waves in (**b**) are marked by arrow heads in (**a**). The respective latencies to the peak effects (broken lines in **b**) are pooled from 6 slices and summarized in (**c**), showing no significant difference (ns) between the peak effects of the 2 drug solutions. $^{**}p<0.05$ (2-tailed Student's paired *t*-test)

This is illustrated by the 2 arrow heads in Fig. 1a, which correspond to the times of peak effect exerted by the 2 drug solutions as revealed by the difference waves in Fig. 2b. We measured the latency-to-peak of each difference wave (length of broken lines in Fig. 1b) in 6 slices and the results showed that adding 2 mM taurine to 10 μM 25-6981 did not change the latency to peak effect (Fig. 1c, $p=0.20$, Students paired *t*-test). In 4 other slices, adding Ro25-6981 (5 μM) to 2 mM taurine (i.e. reversing the order of application) also did not change the latency of peak effect of taurine ($p=0.73$, not illustrated). The finding that the effects of the 2 drugs peak at the same time is consistent with the notion that taurine shares the same cellular target as Ro25-6981.

3.2 *Dose-Dependent Responses of Ro25-6981 in the Absence and Presence of Taurine Reveal Overlapping Rather Than Additive Nature of the Effects of the 2 Drugs*

The magnitude of the effect of a bath-applied drug was measured as the amplitude at the peak of the difference wave. For comparison, the amplitude was normalized to the control response measured at the time the peak occurred. The effect of

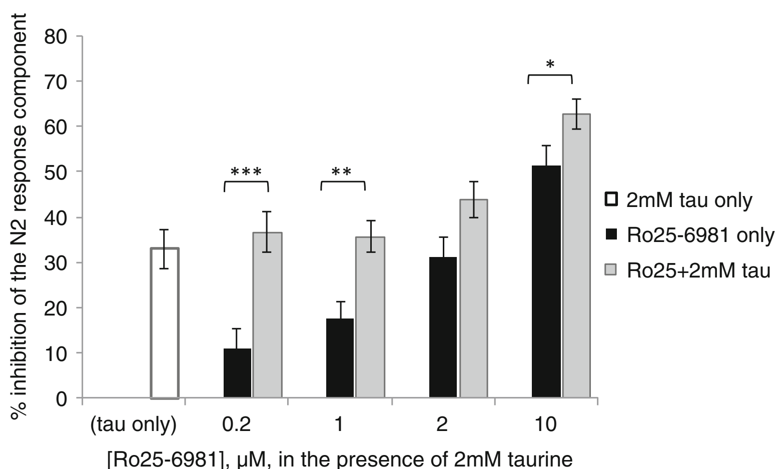

Fig. 2 The inhibition of the N2 response component by 2 mM taurine was progressively replaced by increasing doses of co-applied Ro25-6981: Note absence of additive interaction between the 2 modulators. *p<0.1; **p<0.05; ***p<0.005, Student's 1-tailed unpaired *t*-test

Ro25-6981 was dose-dependent. Non-linear curve-fitting of the effects (using Slidewrite v.4, Advanced Graphics) of 5 doses measured in 27 slices showed an IC_{50} of 2.5 μM and a maximum inhibition of 61 %. At 10 μM, Ro25-6981 inhibited the evoked potential by 51 ± 4 % (SEM, n = 9).

Taurine (2 mM) applied alone decreased the amplitude of the evoked potential by about 32.9 ± 4 % (n = 7, Fig. 2). The combined effect of co-applied 2 mM taurine and Ro25-6981 was dependent on the concentration of Ro25-6981: Low concentrations (<2 μM) of Ro25-6981 combined with taurine yielded an inhibition of N2 similar to the effect of taurine alone; while concentrations above 2 μM combined with taurine produced inhibitions of N2 greater than the taurine effect, but still less than the sum of the 2 individual effects. Figure 2 summarizes the effects of pooled data (n = 7–9 for each drug or combination). The effect (% inhibition of the amplitude of the N2 response component) of Ro25-6981 (dark columns) increased dose-dependently from 0.2 to 10 μM. While co-application with 2 mM taurine (light columns) increased the inhibitory effect of all doses of Ro25-6981, the increases were progressively smaller and less significant for incrementally higher doses of Ro25-6981 (At 10 μM, $p = 0.5$; 1-tailed unpaired *t*-test). Furthermore, at 10 μM Ro25-6981, the combined effect became significantly greater than the effect of 2 mM taurine applied alone ($p < 0.0001$). Pooled data thus reveal a trend of progressive replacement of taurine effect by those of increasing Ro25-6891 doses. This in turn suggests that the 2 modulators share the same molecular target, viz. the GluN1/GluN2B-receptor subtype, even though their sites of action may or may not be identical.

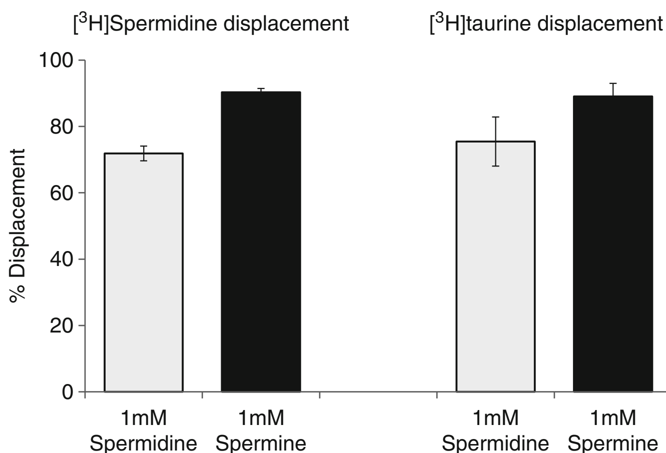

Fig. 3 Polyamines have the ability to displace both spermidine and taurine from cortical tissue. Displacement of [^{3}H]spermidine or [^{3}H]taurine specific binding to cortical membranes by cold spermidine or spermine was measured in binding assays. Data represents the mean and S.E.M. of three independent experiments

3.3 *Radioligand Binding Assay Supports Interaction of Taurine with the NMDA Receptor Polyamine Site*

We further explored if taurine interacts with GluN1/GluN2B subunits of NMDA receptor by conducting binding studies in crude rat frontal cortex membrane homogenate with [^{3}H]spermidine and [^{3}H]taurine. Well washed rat frontal cortical membrane preparations were incubated with 0.25 μM [^{3}H]spermidine or 1 μM [^{3}H]taurine in the presence or absence of cold spermidine and spermine (1 mM) for 30 min at 30 °C. Figure 3 shows that 1 mM spermidine or spermine displaced specific [^{3}H] taurine binding to frontal cortical membranes with comparable strength as their displacement of [^{3}H] spermidine. These data indicate that polyamines and taurine compete or interact at a common binding site either directly or via an allosteric mechanism. Since polyamines bind to a specific site(s) on the NMDA receptor and exhibit higher apparent affinity to GluN1/GluN2B than GluN1/GluN2A, this suggests that taurine's TAG-insensitive receptor is localized on either GluN1 or GluN2B subunit.

3.4 *Chronic (30-Day) Taurine Treatment Increases Membrane Expression of NMDA GluN2B, but Down-Regulates the AMPA GLUR2 Subunit*

To further identify the subunit where taurine interacts with the NMDA receptor, we investigated the expression of NMDA and AMPA receptor subunits in synaptosomal membranes prepared from rat frontal cortex following chronic taurine treatment by

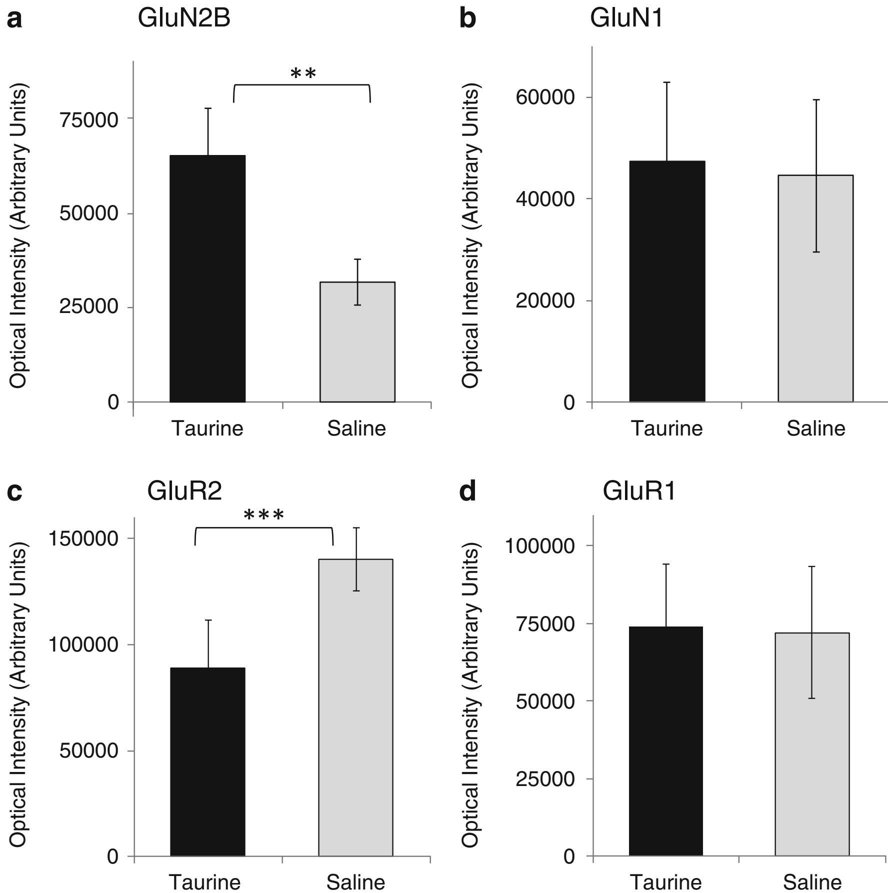

Fig. 4 Effects of chronic (30 daily 100 mg/kg, i.p.) taurine treatment on membrane expression of NMDA and AMPA subunits. (**a**) Chronic treatment increases the expression of NMDA receptor GluN2B subunit (**$p < 0.05$, n = 6, Student's 1-tailed paired *t*-test). (**b**) The same treatment induced no change in the NMDA GluR1 subunit (n = 6). Short term taurine treatment did not cause any detectable change in expression of GluN1, GluN2A or GluN2B subunits (not shown). (**c**) In contrast, long term taurine treatment significantly reduced the membrane expression of AMPA GluR2 subunit (***$p < 0.01$ n = 9 paired *t*-test). (**d**) However, AMPA GluR1 subunit expression was unaffected (n = 10)

using western blotting. Thirty daily i.p. injection of 100 mg/kg taurine significantly increased membrane expression of GluN2B (Fig. 4a, $p < 0.05$, n = 6) without affecting the expression of GluN1 (Fig. 4b, n = 6). Chronic taurine treatment, on the other hand, significantly reduced the synaptic membrane expression of AMPA receptor GluR2 subunit (Fig. 4c, $p < 0.01$, n = 9) without changing the expression of the AMPA receptor subunit GluR1 (Fig. 4d, n = 10).

4 Discussion

Our electrophysiological, radioligand binding and western blotting studies revealed several characteristics of the interaction between taurine and the NMDA receptor. First, Ro25-6981, which selectively blocks the GluN1/GluN2B subtype of the NMDA receptor (Fischer et al. 1997), likely shares the same cellular targets with taurine in the cortical microcircuit architecture, as the effects of both drugs had similar time courses, peaking with the same latency at a late stage of the N2 component of the evoked response. The evoked potential in the cerebral cortex is a patterned composite of responses from many cell groups that are activated in stereotypical temporal sequences (Silberberg et al. 2005; Luczak and Maclean 2012). It follows that the same latencies for the peaks of 2 waveforms can reflect maximum actions at the same cell groups (and the converse would be true if the latencies differ). Furthermore, the observation that the effects of neither taurine nor Ro25-6981 share the same latency with the control response shows that the 2 effects are to some extent group-selective, rather than global and proportionate on all parts of the response. Thus, the data seem to argue against a presynaptic target for the 2 inhibitors, which would tend to exert more global effects on all glutamate receptors.

Second, the present finding that the dose-dependent effect of Ro25-6981 overlaps functionally with taurine in suppressing the glutamate-induced evoked potential in frontal cortical slices suggests that the taurine-mediated inhibitory modulation of NMDA receptor is manifested via an interaction with the GluN1/GluN2B receptor subtype. Although our experiments had not included the use of saturating concentrations of Ro25-6981 to co-apply with taurine (in order to avoid cross activation of the GluN2A receptor), our data clearly show that the effect of taurine co-applied with increasing moderate concentrations (0.2–2 μM) of Ro25-6981 provided no additional inhibitory effect beyond that seen with taurine alone. In contrast, at higher concentrations of Ro25-6981, the combined effect of the 2 drugs appeared to mainly reflect the action of the high dose of Ro25-6981. Thus, the data appears to support the lack of additivity between the actions of Ro25-6981 and taurine, thus suggesting that taurine exerts its effect via the GluN2B receptor subtype. However, an interaction of taurine with the GluN2A subtype cannot be totally ruled out by the present data. We are in the process of testing this possibility with the aid of specific GLuN2A receptor antagonists.

Thirdly, our radioligand binding assay shows that polyamines displace bound taurine from membranes much like their displacement of radiolabeled polyamine. Thus it appears that taurine and polyamines do interact at the same site either through direct competition or allosterically. Since spermine has been shown to preferentially bind to GluN1 and/or GluN2B (Han et al. 2008; Karakas et al. 2009, 2011), its interaction with taurine lends support for a target site for taurine at one or both of these subunits.

The finding of an increased membrane expression of GluN2B subunit with no change in expression level of GluN1 in frontal cortex membranes following chronic taurine treatment further indicates a role for GluN2B in the action of taurine. This

result suggests that chronic inhibition of the GluN2B subunit leads to an increase in cellular subunit concentration which may result from enhanced synthesis or reduced breakdown of the subunit protein that develop during prolong inhibition of the NMDA receptor. At the same time, we identified a significant decrease in the expression of AMPA receptor GluR2 subunit with no significant change in the expression of the GluR1 subunit of the AMPA receptor, suggesting a reciprocal relation between NMDA and AMPA receptor subunits.

One possible mechanism for this intriguing down-regulation of the AMPA GluR2 subunit is that during chronic taurine treatment, excitatory neurotransmission via the NMDA receptor is continuously suppressed, leading to a compensatory enhancement of glutamate neurotransmission via the AMPA receptor, which may in turn down-regulate the expression of GluR2 subunit. An alternative hypothesis involves a process resembling synaptic scaling. Previous investigators have highlighted the possible involvement of GluR2-lacking AMPA receptor in long-term potentiation and depression, synaptic scaling, and cocaine craving (Cull-Candy et al. 2006; Liu and Zukin 2007; Bellone and Luscher 2006; Mameli et al. 2007; Conrad et al. 2008). In particular, synaptic scaling has been described as a form of homeostatic plasticity in which prolonged activity blockade causes enhanced excitatory synaptic transmission that may involve recruitment of the novel form of AMPA receptor that lack GluR2 subunit, which is calcium-permeable.

5 Conclusions

Our present data provide converging lines of evidence to show that the GluN2B subunit of the NMDA receptor contains the active binding site mediating the TAG-insensitive taurine actions. Further tests will resolve the possible role of the NR1 as another site for interaction with taurine. Taurine does not interact with the AMPA receptor, but upon chronic administration it likely induces prolonged inhibition of NMDA receptor-mediated excitatory synaptic transmission. This may have compensatory effects on the AMPA receptor, which may include a recruitment of the calcium-permeable, GluR2-lacking AMPA receptor. Taurine appears to stand out among the multitude of NMDA receptor antagonists by virtue of several properties. First, it is a partial inhibitor of NMDA receptor so that it may block the glutamate-induced excitotoxicity without interfering with normal physiological functions. Second, its ability to open the TAG-sensitive taurine receptor-linked chloride channel further decreases glutamate-induced excitotoxicity such that it tends not to cause neuro-degeneration often seen with most other NMDA-receptor antagonists (Lidsky et al. 1995). Taurine may thus exhibit better clinical properties as a therapeutic agent.

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Experimental Study of Taurine Antitoxic Activity in the Model of Chronic Epinephrine Intoxication

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1 Introduction

Numerous clinical studies have revealed a negative influence of acute stress in human's and animal's organs (Wurtman 2002), while only fragmentary experimental data exist on the effects of chronic stress on the body. Chronic stress leads to attenuation of body resources and development of cardio-vascular, endocrine, nervous, pulmonary and gastro-intestinal diseases, amongst others. The problem of development of new chronic stress correctors exists; therefore investigation of taurine activity during this pathology is perspective line of research. Taurine demonstrates multiple cellular functions including a central role in neurotransmission, as a trophic factor in CNS development, in maintaining the structural integrity of the membrane, in regulating calcium transport and homeostasis, as an osmolyte, as a neuromodulator and as a neuroprotectant (Wu and Prentice 2010). Our previous experiments have demonstrated neuroprotective and antihypoxic activity of taurine administered in vivo at a dose of 50 mg/kg in different models of brain ischemia (Oleynikova et al. 2009; Makarova et al. 2014). Therefore, we decided to investigate taurine at the above-mentioned dose on the metabolism in rats in a model of chronic epinephrine intoxication. In this work we concentrated our attention on brain metabolism and brain lipid peroxidation as the main pathway of oxidative stress.

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2 Methods

2.1 Experimental Materials and Methods

Experiments were done on male outbred rats (250–300 g). To develop chronic epinephrine intoxication adrenalin hydrochloride was administered intraperitoneally at a dose of 500 mg/kg once daily for a 6 week duration. Two weeks from the experiment start taurine was administered intraperitoneally in a dose of 50 mg/kg in normal saline once daily to the experimental group (n = 8). The control group was treated with the same volume of the normal saline (n = 8). After 28 days of treatment, arterial blood was taken from the right common carotid artery and venous blood was taken from the sagittal sinus under chloral hydrate general anesthesia (300 mg/kg). The following biochemical parameters were assessed to evaluate metabolic changes: total lipids, total cholesterol, total albumin, calcium, medium-sized peptides. To evaluate cerebral lipid peroxidation animals were decapitated under chloral hydrate general anesthesia (300 mg/kg) and brains were extracted. Brains were rapidly bathed in the normal saline; hemispheres were split along the midline. Amounts of malondialdehyde and diene conjugates were measured in the left and right hemispheres.

Total lipid plus total cholesterol concentrations in the blood serum were measured by colorimetric method (reaction with acetic oxide and sulfuric acid for cholesterol; and reaction with vanillin and phosphoric acid for total lipids). Total albumin was measured by biuret method. Ca^{2+} concentration in the blood serum was measured by colorimetric method (reaction with cresol phthalein). Medium-sized peptides were measured by high molecular weight protein settling method (with perchloric acid and ethanol). Diene conjugates concentration was measured in the complex “heptane-isopropyl alcohol.” To evaluate amounts of malondialdehyde, the reaction with thiobarbituric acid was used.

2.2 Statistic Analysis

Statistical significance was determined by Student’s t-test. Each value was expressed as the mean ± SEM. Differences were considered statistically significant when the calculated P value was less than 0.05.

3 Results

Concentrations of total lipids in the control group of animals was elevated up to 31.5 % in the arterial blood and was decreased up to 49.2 % in the venous blood compared to intact animals (Fig. 1). Concentration of total cholesterol was

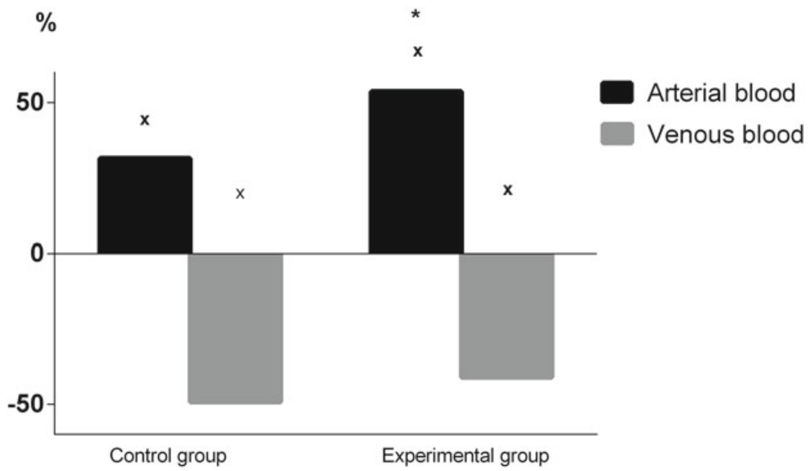

Fig. 1 Taurine administrartion influence on the concentration of total lipids in serum during chronic epinephrine intoxication (% compared with intact animals). p<0.05 compared to: * - Control group, x - Intact animals

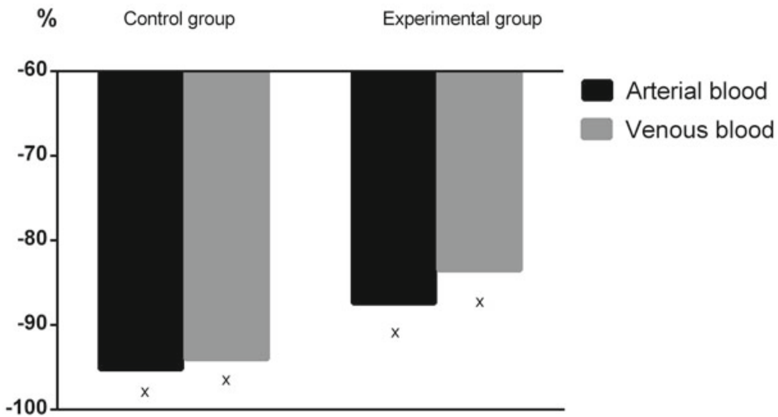

Fig. 2 Taurine administrartion influence on the cholesterol concentration in serum during chronic epinephrine intoxication (% compared with intact animals). p<0.05 compared to: * - Control group, x - Intact animals

significantly decreased both in the venous and arterial blood up to 90.2 % and 94 % respectively (Fig. 2).

Taurine treatment elevated concentration of total lipids up to 53.7 % in the arterial blood and decreased up to 41 % in the venous blood compared to intact animals. Concentration of total cholesterol did not differ significantly compared to control group.

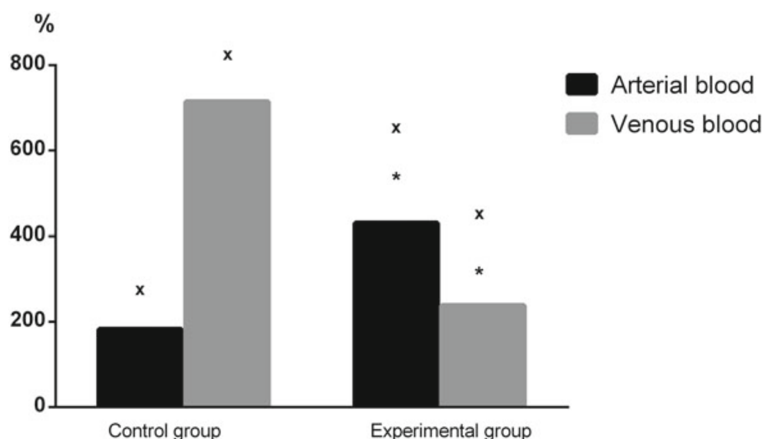

Fig. 3 Taurine administrartion influence on the Ca2+ concentration in serum during chronic epinephrine intoxication (% compared with intact animals). $p<0.05$ compared to: * - Control group, x - Intact animals

Ca^{2+} concentration in blood serum is a well-known biomarker of body metabolism and plays an important role in the cell injury. Significant hypercalcemia was found in the control group of animals. Ca^{2+} concentration in the venous blood was four times higher than in the arterial blood. Animals in the control group had several neurological signs due to this hypercalcemia: weakness and stupor. Taurine treatment blocked development of hypercalcemia: Ca^{2+} concentration was two times higher in the arterial blood and significantly lower (three times lower) in the venous blood (Fig. 3).

Total albumin in the arterial blood was elevated up to 21.8 % in the control group compared to intact animals. Concentration in the venous blood did not differ significantly from the intact group. Taurine administration did not considerably influence this parameter (Fig. 4).

Medium-sized peptides concentration (markers of endogenous epinephrine intoxication) was significantly increased in the venous blood—up to 185.3 % compared to intact group. It was found out that therapeutic administration of taurine reduced medium-sized peptides concentration in the venous blood. Taurine also reduced medium-sized peptides concentration in the arterial blood compared to intact group (Fig. 5).

Next, we investigated taurine's influence on the peroxide oxidation products concentration in the brains. In the control group peroxide oxidation primary products (diene conjugates) were significantly elevated—up to 195.8 % in the right hemisphere and up to 60 % in the left hemisphere. Whereas peroxide oxidation secondary products concentration was decreased up to 38.3 % and 16 % in

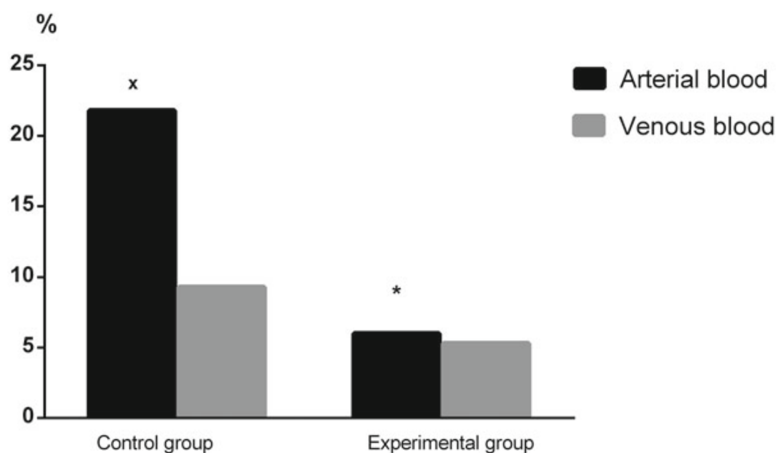

Fig. 4 Taurine administrartion influence on the concentration of total albumin in serum during chronic epinephrine intoxication (% compared with intact animals). p<0.05 compared to: * - Control group, x - Intact animals

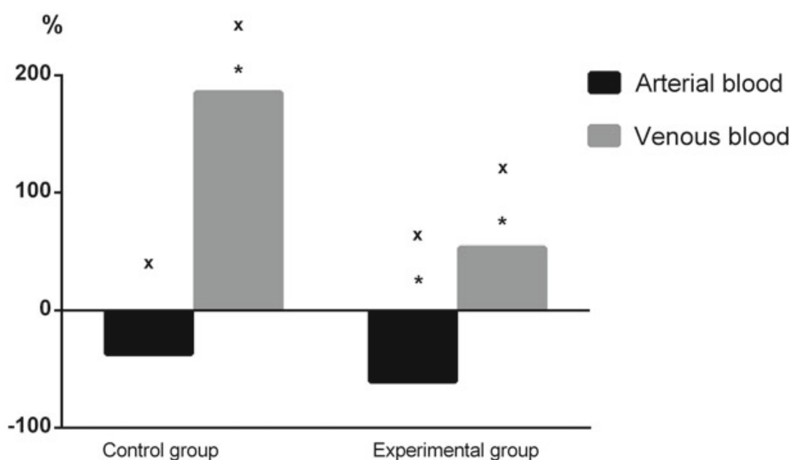

Fig. 5 Taurine administrartion influence on the medium-sized peptides concentration in serum during chronic epinephrine intoxication (% compared with intact animals). p<0.05 compared to: * - Control group, x - Intact animals

the right and left hemispheres respectively compared to intact group. Taurine reduced amount of diene conjugates (2–3 times) and significantly reduced amount of peroxide oxidation secondary products in the both hemispheres compared to control group (Figs. 6 and 7).

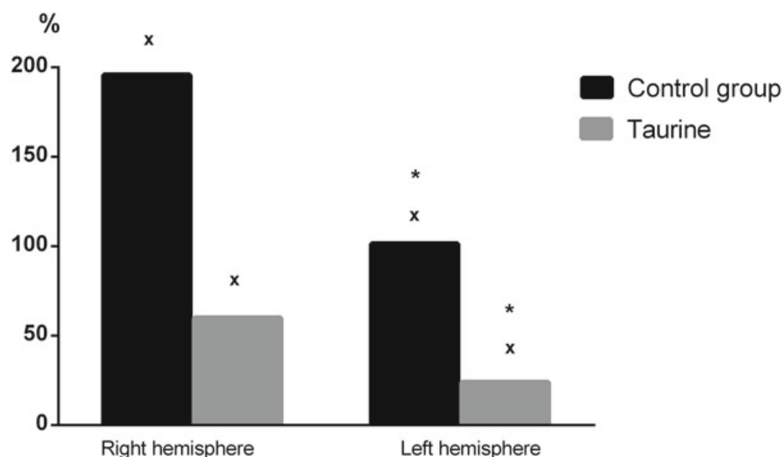

Fig. 6 Taurine administrartion influence on the diene conjugates concentration in the left and right hemispheres during chronic epinephrine intoxication (% compared with intact animals). $p < 0.05$ compared to: * - Control group, x - Intact animals

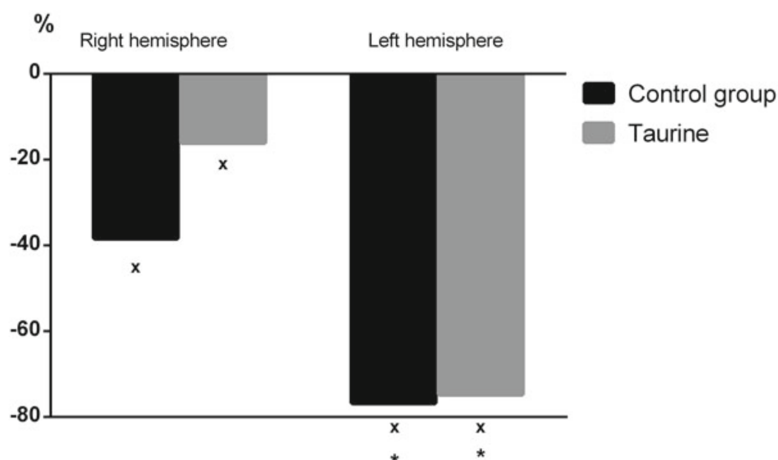

Fig. 7 Taurine administrartion influence on the peroxide oxidation secondary products concentration in the left and right hemispheres during chronic epinephrine intoxication (% compared with intact animals). $p < 0.05$ compared to: * - Control group, x - Intact animals

4 Discussion

According to literature data, emotional stress is the main reason of many human diseases such as cardio-vascular diseases, endocrine, nervous, pulmonary and gastro-intestinal disorders, immune system deteriorations etc. (Loucks et al. 2003; Norberg et al. 2007). During emotional stress epinephrine in the blood vessels

oxidizes to adrenochrome with formation of superoxide radical which is very active and can cause cell apoptosis (Troshin et al. 2006). Cardiotoxicity and neurotoxicity of products of adrenaline oxidation are widely described in the literature (Kolpakov 1974; Rump et al. 2001).

Taurine is one of the most abundant free amino acids in the mammalian body. A number of experimental studies have proved positive antioxidant effect of taurine in the animals (Makarova et al. 2014). Our studies revealed evident antioxidant and antitoxic effects of taurine during excessive adrenochrome formation. We determined that therapeutic administration of taurine dose of 50 mg/kg in the model of chronic epinephrine intoxication reduced medium-sized peptides concentration in the venous blood. Taurine reduced amount of diene conjugates and significantly reduced amount of peroxide oxidation secondary products in the both hemispheres compared to control group. Taurine also blocked development of hypercalcemia and reduced concentration of total lipids in the cerebral venous outflow blood. Taurine did not significantly influence other experimental metabolic parameters.

The antioxidant activity of taurine could be explained by several sites of activity. It is known that taurine reduced oxidative stress in the neutrophiles culture due to neutralization of hypochlorus acid and formation of N-chlortaurine (Nefedov 1999). Moreover, taurine effectively attenuated the hyperhomocysteinemia-induced ROS production and inhibition of Mn-superoxide dismutase and catalase activities in the myocardial mitochondria (Chang et al. 2004). It was shown also that taurine can protect against H_2O_2-induced cell injury in PC12 cell cultures by reducing H_2O_2 -induced endoplasmatic reticulum stress (Pan et al. 2010). In addition, taurine plays an important role in reducing endoplasmatic reticulum stress in C2C12 and 3T3L1 cells (Song et al. 2009).

5 Conclusion

Therapeutic administration of taurine in the dose of 50 mg/kg reduced cerebral metabolic disorders and prevented cerebral lipid peroxidation during chronic epinephrine intoxication.

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Part V
Taurine in Cardiovascular and Renal Systems

Does Taurine Prolong Lifespan by Improving Heart Function?

Stephen W. Schaffer, K.C. Ramila, Chian Ju Jong, Aza Shetewy, Kayoko Shimada, Takashi Ito, Junichi Azuma, and Eugene Cioffi

1 Introduction

Taurine is an abundant amino acid that is essential for the cat but semi-essential for man (Knopf et al. 1978; Gaull 1986). Taurine is synthesized from cysteine in the liver. While the biosynthesis of taurine is depressed or absent in both cats and humans, the turnover of taurine is dramatically increased in the cat, causing a loss of large amounts of taurine in the feces as taurocholic acid. By contrast, the conjugation of bile acids in man involves either glycine or taurine; glycine substitutes for taurine when intracellular taurine levels are very low (Knopf et al. 1978). Because the combination of extremely low rates of taurine biosynthesis and high rates of taurine loss from the body as taurine conjugated bile acids, the cat is dependent upon a dietary source of taurine to maintain bodily taurine stores (Knopf et al. 1978). In contrast to the cat and man, rodents synthesize abundant amounts of taurine in the liver, which insulates the rodent from dietary-induced taurine deficiency (Huxtable and Lippincott 1982).

Recently it has been shown that taurine deficiency diminishes lifespan in mice lacking the taurine transporter, a phenomenon the authors attributed to accelerated aging. However, taurine deficiency is also associated with severe disruption of cellular function and in some cases cell death. There is also evidence that taurine attenuates pathological events that are potentially fatal. Thus, the effect of taurine deficiency on lifespan is a complex phenomenon, involving both physiological dysfunction and premature aging. Because the effect of taurine on lifespan is a new

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concept, the present review focuses on potential mechanisms underlying the reduction in lifespan, with special emphasis on contractile dysfunction, mitochondrial disruption, oxidative stress and inflammation.

2 Characteristics of Taurine Depletion

2.1 Pathological Changes Mediated by Taurine Depletion

The levels of taurine in two taurine-sensitive tissues, the heart and the brain, are significantly higher in the newborn than in the adult (Sturman 1986), however, taurine levels increase in the retina during development (Lombardini 1991). Taurine content of the two tissues are also species-dependent. Kocsis et al. (1976) found that the higher the heart rate of a given species, the greater the cardiac taurine content; hence rat hearts (30.5 μmol/g wet wt) contain the most taurine followed by cat hearts (11.6 μmol/g wet wt), and human hearts, which contain the least taurine. Similarly, brain taurine levels are the highest in the rat (4.4 μmol/g wet wt) followed by the cat (2.3 μmol/g wet wt) and finally man (1.4 μmol/g wet wt) (Sturman 1986). Although the size of body taurine in man is less than that in other species, humans are resistant to the development of taurine deficiency. Interestingly, rodents, whose body taurine levels are very high, are also resistant to taurine deficiency. Therefore, no direct correlation exists between body taurine levels and the dietary taurine requirement of a given species. Rather, the reliance of a species on dietary taurine to maintain body taurine levels depends upon the rate of taurine biosynthesis by the liver and the rate of taurine loss from the body as conjugated bile acids. Certain species, such as cats, fox, certain dogs, certain primates and giant anteaters, become taurine deficient if fed a taurine free diet, as they are incapable of readily synthesizing taurine in the liver. Moreover, they utilize taurine exclusively in the conjugation of bile acids (Knopf et al. 1978). Although man does not readily synthesize taurine in the liver, humans can substitute glycine for taurine in the bile acid conjugation reaction, thereby preventing significant loss of taurine during periods of reduced dietary taurine intake. Thus, man does not readily develop taurine deficiency (Gaull 1986).

Knopf et al. (1978) initially defined taurine as an essential nutrient in the cat, as kittens fed a casein diet for 7.5 weeks lose 99 % of their hepatic taurine, 93 % of cardiac taurine, 45 % of retinal taurine and 90 % of cerebellar taurine content. In an earlier study by the same group, cats fed a taurine deficient diet for 24 weeks lost 80 % of retinal taurine, a condition that led to disintegration of the photoreceptor outer segment lamellae of the retina (Hayes and Carey 1975). In a follow-up study, Schmidt et al. (1977) found that cats fed a taurine deficient diet up to 10–45 weeks lost 50 % of their retinal taurine content, with the variability in response attributed to the time required to deplete hepatic taurine content. They also found that cats that lost more than 50 % retinal taurine developed a retinopathy characterized by diminished rod and cone ERG amplitudes and in some cases sight was lost. More recently, it has been shown that mice lacking the taurine transporter also develop a taurine deficiency retinopathy (Heller-Stilb et al. 2002; Rascher et al. 2004).

2.2 Taurine Deficiency Leads to Fatal Cardiomyopathy

Taurine deficiency is also associated with the development of dilated cardiomyopathy (Pion et al. 1987; Ito et al. 2008). According to Fox and Sturman (1992) the mean myocardial taurine content of cats fed a diet containing 0.05 % taurine is 12.0 ± 2.3 μmol/g wet wt compared to 3.75 ± 1.99 μmol/g wet wt for cats suffering from taurine deficient cardiomyopathy. The University of California, Davis Veterinary Medical Teaching Hospital discovered that of cats presenting at their institution between October 1986 and September 1988, 100 % suffering from dilated cardiomyopathy showed at least some taurine deficiency (Pion et al. 1992). Moreover, among cats treated for dilated cardiomyopathy with taurine, 58 % survived while only 13 % of the cats with idiopathic dilated cardiomyopathy survived. This finding is consistent with the observation that most cases of feline dilated cardiomyopathy are reversed with taurine therapy and that untreated taurine deficient cardiomyopathy is fatal. Interestingly, although classical heart failure therapy (using diuretics and vasodilators) provides some clinical benefit, it does not prevent the death of taurine deficient cats, most of which succumb within 6 months of cardiomyopathy diagnosis. It is relevant that fatalities caused by taurine deficiency are not restricted to feline dilated cardiomyopathy, as taurine deficient cardiomyopathy is also a cause of death among certain dogs (Kittleson et al. 1997; Belanger et al. 2005).

The taurine deficient feline heart is characterized by diminished contractility, as evidenced by decreases in left ventricular pressure and ±dP/dt (maximal rate of left ventricular pressure rise and fall) (Novotny et al. 1991). It also undergoes a shift in the diastolic pressure-volume relationship, supporting the idea that as a result of volume overload the ventricular chamber of the taurine deficient heart dilates. These changes arise as the heart undergoes ventricular remodeling following an initial insult that reduces contractile function. In the case of the taurine deficient heart, the initial insult is the reduction in complex I activity of the heart (Jong et al. 2012). Not only is the electron transport chain of the taurine deficient heart incapable of rapidly generating adequate amounts of ATP to drive contraction, but the damaged electron transport chain begins producing excessive amounts of reactive oxygen species (ROS), which cause significant mitochondrial oxidative damage while interfering with other important physiological events within the cell, such as Ca^{2+} transport. The initial decrease in contraction is likely related to insufficient ATP and Ca^{2+} for contraction.

In response to the mitochondrial defect, the heart triggers a cascade of events that lead to heart failure. Baroreceptors are activated, leading to the release of catecholamines. The increase in plasma norepinephrine levels, coupled with reduced blood flow, stimulates the renin-angiotensin II-aldosterone system. Angiotensin II is a potent vasoconstrictor while aldosterone acts to retain fluid and salt. As a result, total blood volume and venous return increase, end diastolic pressure rises and ventricular wall stress worsens. Several events (neurohumoral, norepinephrine and angiotensin II, stimulation; ventricular wall stress and the death of some cardiomyocytes via apoptosis) then combine to promote ventricular remodeling. In the taurine deficient heart, ventricular remodeling causes eccentric myocardial hypertrophy

and dilatation of the chamber wall. These events eventually trigger the onset of congestive heart failure. It is significant that normal levels of taurine not only prevent the mitochondrial changes that initiate the heart failure cascade but they also interfere with the actions of norepinephrine and angiotensin II, mediators of remodeling (Ito et al. 2014).

2.3 Impaired Protein Phosphorylation and Ca^{2+} Handling Contribute to Development of Taurine Deficient Cardiomyopathy

Diminished Ca^{2+} transport by the sarcoplasmic reticulum (SR) contributes to the weakening of cardiac muscle and to the reduction in contractile function of the failing heart. In the normal contraction-relaxation cycle, the SR Ca^{2+} ATPase pumps Ca^{2+} from the cytosol into the sarcoplasmic reticular vesicles, an event that serves two functions. First, it reduces cytosolic Ca^{2+} concentration below the Km for Ca^{2+} binding to the muscle protein, troponin, thereby triggering muscle relaxation. Second, the loading of the SR vesicles with Ca^{2+} ensures a ready supply of Ca^{2+} for initiation of contraction when the heart is stimulated. Thus, declines in the activity of the SR Ca^{2+} ATPase leads to both diminished contractility and relaxation.

Abnormal Ca^{2+} handling by the failing heart is thought to contribute to the severity of heart failure (MacLennan and Kranias 2003). In support of that idea, Beeri et al. (2010) found that ventricular remodeling in the post-myocardial infarcted heart is associated with the downregulation of the SR Ca^{2+} ATPase. However, contractile function improves and the degree of ventricular remodeling decreases when the ischemia-reperfusion insult is performed on hearts containing elevated SR Ca^{2+} ATPase content.

Figure 1 shows that SR Ca^{2+} ATPase activity is decreased in the taurine deficient heart (Ramila et al. 2015). However, unlike many other models of heart failure, the decrease in SR Ca^{2+} ATPase activity of the taurine deficient heart is not caused by a decrease in enzyme expression (data not shown). Rather, enzyme activity is significantly decreased only at Ca^{2+} concentrations that are physiologically relevant (~0.3 μM) and that influence the relaxation phase of the Ca^{2+} transient. One of the key regulators of SR Ca^{2+} ATPase activity is the phosphoprotein, phospholamban, which interacts with the SR Ca^{2+} ATPase to form a complex with reduced Ca^{2+} affinity for the ATPase (MacLennan and Kranias 2003). However, phosphorylation of phospholamban by Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII) weakens the interaction between phospholamban and the ATPase, thereby increasing SR Ca^{2+} ATPase activity.

Taurine deficiency appears to prevent this series of events from activating the SR Ca^{2+} ATPase. Central to the actions of taurine deficiency is modification of the regulatory enzyme, CaMKII, which is commonly expressed as a multimeric protein with 12 subunits (Hoelz et al. 2003). Each monomer has three domains, with the

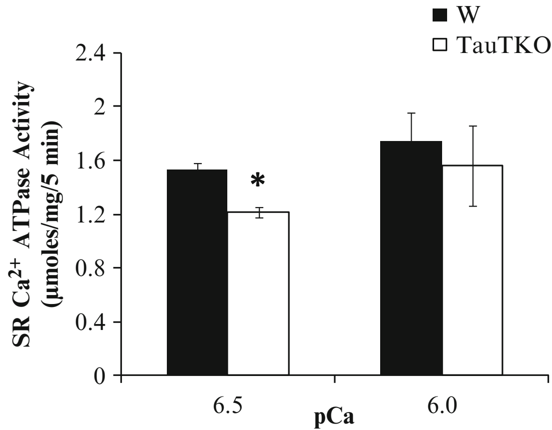

Fig. 1 Reduction in sarcoplasmic reticular Ca^{2+} ATPase activity of TauTKO heart. Enriched sarcoplasmic reticulum (SR) was prepared from wild-type (W) and taurine transporter knockout mouse (TauTKO) hearts. SR Ca^{2+}-dependent ATPase was determined in Tris buffer supplemented with oxalate and azide and containing either 0, 3.2×10^{-7} M or 10^{-6} M Ca^{2+}. Data shown represent means ± S.E.M. of 4–5 different preparations. The asterisk denotes a significant difference between wild-type and TauTKO hearts ($p < 0.05$). Permission for the use of this figure has been provided by the American Physiological Society (Ramila et al. 2015)

C-terminal domain directing the assembly of the multimer; the N-terminal catalytic domain binds to potential substrates and directs the serine/threonine kinase function of CaMKII. In the intervening linker region lie the regulatory domain with two primary roles. This regulatory domain acts as a substrate for the catalytic domain within each CaMKII monomer, whilst adjacent regulatory domains within the multimer block both ATP and substrate binding to the catalytic domain itself (Rosenberg et al. 2005). Overall, the catalytic and regulatory domains are tightly associated under basal conditions, resulting in autoinhibition of the kinase. The regulatory domain binds calmodulin with a K_D of 10–70 nM when intracellular [Ca^{2+}] is elevated (Gaertner et al. 2004). When calmodulin binds to CaMKII, the associated domains are disrupted, exposing the catalytic domain for substrate binding and abrogating autoinhibition of the kinase.

Due to the relationship between the structure and function of CaMKII kinase, CaMKII is able to translate a diverse set of signaling events into downstream physiological effects. Prolonged Ca^{2+} cycling activates the kinase and promotes post-translational modifications that are dependent upon the biochemical environment of the heart (Erickson 2014); these result in sustained, chronic CaMKII activation which can be pathological. Conditions of prolonged Ca^{+2}/calmodulin association with CaMKII, allow for intersubunit autophosphorylation of CaMKII monomers at Thr286/287, with two important effects. First, the binding affinity of

calmodulin for the CaMKII regulatory domain increases >1,000-fold (Meyer et al. 1992). Second, the negatively charged phosphate at Thr286/287 precludes reassociation of the regulatory and catalytic domains, preventing autoinhibition even if $[Ca^{2+}]_i$ falls and calmodulin and CaMKII dissociate (Erickson 2014). In addition to phosphorylation, other post-translational modifications can occur on CaMKII such as *O*-GlcNAcylation.

The hexosamine biosynthesis pathway generates uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc), which is the precursor for both N- and O-linked glycosylation of proteins, and the substrate for β-*O*-linked N-acetylglucosamine (*O*-GlcNAc) modification of proteins (Yamamoto and Yamamoto 2015). In contrast to other glycation reactions, *O*-GlcNAc glycosylation is a reversible and labile post-translational modification, often competing with phosphorylation events and pathways in key proteins (Zeidan and Hart 2010). UDP-GlcNAc is transferred onto target proteins by *O*-GlcNAc transferase (OGT), and is catalytically removed by *O*-GlcNAcase (OGA). *O*-GlcNAc and O-phosphate exhibit a complex interplay in signaling, transcriptional, and cytoskeletal regulatory proteins within the cell. In fact, a major phosphate-removing enzyme, protein phosphatase 1, is in a dynamic complex with OGT, indicating that the same enzyme complex removes both O-phosphate and concomitantly attaches *O*-GlcNAc (Varki et al. 2009). *O*-GlcNAcylation represents a metabolically-derived, ubiquitous and recruitable stress sensor, and as such, offers cardioprotection from various lethal stressors (Zanfir et al. 2012). *O*-GlcNAc modification of CaMKII at Ser279 activates CaMKII. The CaMKII-dependent regulation of SR Ca^{2+} movement is prevented by pharmacological inhibition of *O*-GlcNAc signaling (Erickson et al. 2013). Although the effect of taurine deficiency on the hexosamine pathway has not been examined, Mozaffari et al. (1986) found that taurine deficiency stimulates cardiac glucose uptake and glycolysis, with most of the glucose flowing through the glycolytic pathway being converted to lactate. Because taurine deficiency reduces respiratory chain activity, flux of substrate through glycolysis is limited by the accumulation of NADH. Feedback inhibition by NADH creates a bottleneck, which allows some of the entering glucose to be diverted into the hexosamine pathway, providing a source of UDP-GlcNAc for *O*-GlcNAc glycosylation.

As seen in Fig. 2, the activity and phosphorylation state of CaMKII, as well as the phosphorylation state of phospholamban, are decreased in the taurine deficient heart. Because the levels of protein phosphatase 1 are elevated in the taurine deficient heart, it is likely that protein phosphatase 1 catalyzes the dephosphorylation of the autophosphorylation site at Thr286/287. Although not determined, it is also likely that the removal of the phosphate group on Thr286/287 would be replaced by an *O*-GlcNAc modification, which in turn could possibly contribute to further depression of CaMKII activity. As seen in Fig. 2, depressed CaMKII activity results in diminished phosphorylation of phospholamban at Thr17, which in turn strengthens the inhibitory interaction of phospholamban and SR Ca^{2+} ATPase. We propose that this defect contributes to a worsening of the cardiomyopathy, which is potentially fatal.

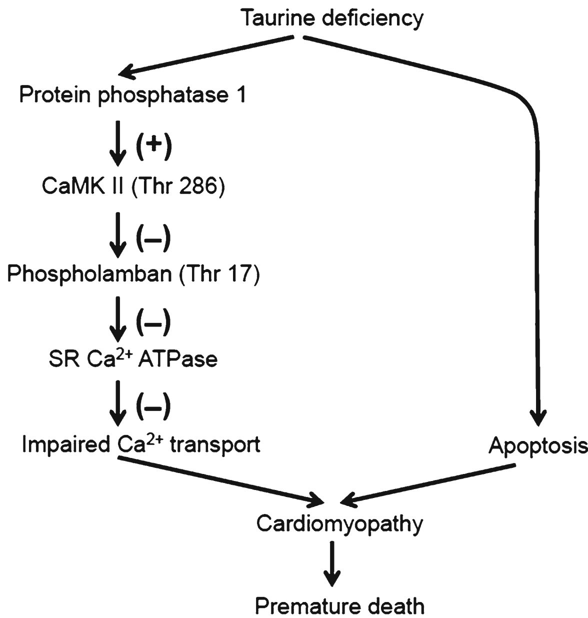

Fig. 2 Development of taurine deficient cardiomyopathy. Taurine deficiency is associated with an elevation in protein phosphatase 1 activity. The phosphatase dephosphorylates Thr286/287 of CaMKII, a modification that decreases enzyme activity. The phosphorylation state of Thr17 of phospho-lamban, which is a substrate of CaMKII, declines allowing the formation of the phospholamban/SR Ca^{2+} ATPase complex, decreasing transporter activity and suppressing diastolic function. Taurine deficiency also promotes apoptosis, which together with the decrease in $[Ca^{2+}]_i$, leads to the development of cardiomyopathy, which can be fatal

3 Taurine Depletion Accelerates Aging

3.1 Telomere Hypothesis of Aging

Aging is a complex, degenerative process that leads to a decline in physiological functions within multiple tissues, but in particular in tissues with a high demand for energy, such as the heart. Although damaged organelles and macromolecules accumulate in aged tissues, the processes involved in clearing dysfunctional cellular components are defective. Moreover, aging is associated with reduced tolerance to stress, which together with other characteristics of aging, increases the risk of morbidity and mortality (Martin et al. 1996; Kirkwood and Austad 2000; Rattan 2006; Vijg and Campisi 2008; Carnes 2011).

The recognition that cells have a finite lifespan dramatically influenced the study of aging (Hayflick and Moorhead 1961). In the 1970s, Olovnikov (1996) proposed that shortening of telomeres, which are stretches of repetitive DNA strands that cap the ends of chromosomes, explains the finite lifespan of cells in culture. However, it

wasn't until 1990 that Harley et al. (1990) observed a reduction in telomeric DNA content of aging fibroblasts. Because the end of the chromosome is close to the end tandem repeats of the telomere, it was thought that telomere shortening might stall DNA replication. In tumor cells, telomere shortening is associated with the induction of apoptosis (Karlseder et al. 1999; Hahn et al. 1999). Furthermore, in normal human cells overexpression of telomerase, an enzyme that re-elongates telomeres, prolongs the lifespan of those cells (Bodnar et al. 1998) although other investigators have suggested that the mechanism by which telomerase prolongs lifespan may not involve a change in telomere length.

Telemore shortening and premature aging can be induced by a variety of mild stresses that irreversibly inhibit growth, alter cell morphology, modulate gene expression and promote the upregulation of p53 or p21 (von Zglinicki 2002). Telemores contain a high content of guanine, which is readily damaged by oxidative stress, alkylation and UV irradiation. Because the telomeres are capped, the classical DNA repair enzymes do not readily recognize oxidative DNA damage of the telomere. Thus, the frequency of single strand breaks is greater in telomeres than in the rest of the chromosome. Thus, a good strategy for reducing the rate of telemore shortening and the appearance of senescent cells is treatment with antioxidants and free radical scavengers.

3.2 Mitochondrial Theory of Aging

It has been proposed that mitochondria, by serving as the major source of reactive oxygen species (ROS) in most cell types, play a central role in senescence (Harman 1983). According to the mitochondrial theory of aging, the aging process leads to oxidation of mitochondrial DNA, resulting in DNA damage (i.e. base modifications, strand breaks and both deletion mutations and point mutations). These DNA changes result in defects in the synthesis of mitochondria encoded proteins, which are required for normal respiratory chain function (Hiona and Leeuwenburgh 2008; Ma et al. 2009; Wang et al. 2013). Besides adversely affecting ATP production, impaired respiratory function in aged tissue leads to excessive ROS production.

The two major sources of mitochondrial ROS are complexes I and III, with complex I generating and releasing superoxide anion directly into the mitochondrial matrix and with complex III generating and releasing superoxide anion into either side of the inner mitochondrial membrane. Point mutations in either complex or treatments with respiratory chain inhibitors increase superoxide production and decrease lifespan, as electrons are diverted from the respiratory chain to oxygen, forming superoxide anion (Stockl et al. 2007; Ma et al. 2009; Ishii et al. 2011). According to some investigators, this sequence of events sets up a "vicious cycle" in which the generation of ROS leads to further oxidative damage of mitochondrial DNA, causing greater respiratory chain dysfunction and the completion of the cycle with the generation of even more ROS (Hiona and Leeuwenburgh 2008; Wang et al. 2013). Although the role of the "vicious cycle" in aging is controversial, it is widely accepted that mitochondrial dysfunction particulates in the aging process.

Particularly noteworthy is the observation that there is both a reduction in the number of mitochondria in tissues, such as heart, and structural changes in existing mitochondria during aging (Herbener 1976). Also, useful information on aging has been gleaned from a homozygous knock-in mouse, known as the mtDNA mutator mouse, which contains a proofreading deficient copy of the catalytic subunit of mitochondrial polymerase gamma. Trifunovic et al. (2005) concluded that the primary inducer of premature aging in the polymerase gamma deficient mice is respiratory chain dysfunction rather than ROS production. However, consistent with the importance of oxidative mitochondrial DNA damage in the aging process, Kolesar et al. (2014) recently observed similar mitochondrial oxidative damage in the mtDNA mutator mouse and in older adults. Knocking out specific antioxidant enzymes also decreases lifespan while overexpression of some of the antioxidant enzymes prolongs lifespan (Muller et al. 2007).

3.3 Effect of Taurine Deficiency on Mitochondrial Function

Taurine deficiency mediates a similar series of events as the complex I inhibitor, rotenone. In both conditions, activity of complex I is reduced, superoxide anion is generated and the aging process is initiated. However, the mechanism by which both conditions cause a reduction in complex I activity differs. Taurine forms a complex with the wobble position uridine of $tRNA^{Leu(UUR)}$, a modification that strengthens the interaction of the UUG codon with the AAU* anticodon, where U* represents the conjugated product, 5-taurinomethyluridine (Kurata et al. 2008). Taurine deficiency diminishes the availability of taurine for the conjugation reaction. As a result, the synthesis of the mitochondria encoded protein (ND6), whose mRNA contains the most UUG codons (8), is diminished and ND6 protein content of the taurine deficient cardiomyocyte declines (Jong et al. 2012). Because ND6 is a key subunit of complex I, the activity of complex I declines and respiratory chain function is impaired (Jong et al. 2012). In addition, despite the reduction in the delivery of fewer electrons to the respiratory chain, ND6 deficiency is also associated with a diversion of electrons from complex I to oxygen forming superoxide anion (Jong et al. 2012). Although the mechanism by which excessive mitochondrial superoxide production contributes to senescence has not been determined, it is generally accepted that elevations in ROS transform the cell through mutations, accumulation of damaged macromolecules, excessive apoptosis and altered cell signaling, events involved in aging (Ishii et al. 2011).

3.4 Link Between Aging and Inflammation

Another mechanism that appears to contribute to the process of senescence is inflammation (Chung et al. 2009). Two observations link inflammation and aging: (1) mediators of inflammation are elevated in aging-associated diseases and (2)

ROS, which underpin the process of aging, regulates transcription factors, such as NF-κB, and controls the production of a host of pro-inflammatory mediators. According to Helenius et al. (1996), levels of NF-κB are elevated in the aged heart, as ROS activate upstream signaling kinases, such MAPK and IκB kinase, which in turn increase the levels of NF-κB (Zandi et al. 1997). Taurine acts through two mechanisms to attenuate the aging process. First, by preventing the generation of ROS by the mitochondria, taurine diminishes the activation of the redox sensitive protein kinases, MAPK and IκB kinase. Second, taurine treatment increases taurine chloramine production, which in turn inhibits the activation of NF-κB by oxidizing IκB-α at Met45 (Kanayama et al. 2002; Schuller-Levis and Park 2003). Downstream from NF-κB, taurine chloramine inhibits cytokine production and the generation of other inflammatory mediators (Marcinkiewicz et al. 1998; Kontny et al. 2000). In cats, taurine deficiency is associated with leukopenia, an increase in mononuclear leukocytes, diminished respiratory burst of polymorphonuclear cells and a reduction in serum gamma globulin, all indications of immunologic deficiency (Schuller-Levis et al. 1990). Similarly, the TauTKO mouse exhibits immune deficiency, as indicated by impaired ability to resist infections with *Plasmodium chabaudi* malaria (Delic et al. 2010). Because all of the taurine deficient mice die from the infection while ~90 % of the wildtype mice survive the infection, the sensitivity to infections could represent another mechanism underlying the reduction in lifespan.

4 Taurine Depletion Mimics Aging

4.1 Effect of Taurine Deficiency and Aging on ER Stress

When misfolded or unfolded proteins accumulate in the endoplasmic reticulum, the cell responds by stimulating the degradation of those proteins by the process known as unfolded protein response (UPR) (Malhotra and Kaufman 2007). Because the accumulation of the abnormal proteins stresses the endoplasmic reticulum (ER), failure of UPR triggers pro-death signaling pathways (Jager et al. 2012). Among the three transmembrane sensor proteins that participate in UPR, protein kinase R-like ER protein kinase (PERK) mediates a signaling cascade that is activated as an early event in ER stress and plays a key role in both regulating the transcription of stress response genes (Back et al. 2009; Harding et al. 2000) and promoting both an antioxidative stress response and apoptosis (Minamino and Kitakaze 2010). One of the intermediate steps in the PERK-mediated cascade is the phosphorylation and activation of eIF2α by PERK, a process implicated in the reduction in global protein synthesis and the enhancement of mRNA translation of several key proteins involved in cell survival (Back et al. 2009).

Aging is associated with (a) a decline in the activity of the PERK-mediated cascade, (b) diminished phospho-PERK and phospho-eIF2α levels, (c) increased oxidative stress, (d) reduced pro-survival signaling and (e) diminished general translation (Hussain and Ramaiah 2007; Brown and Naidoo 2012). Like aging, taurine

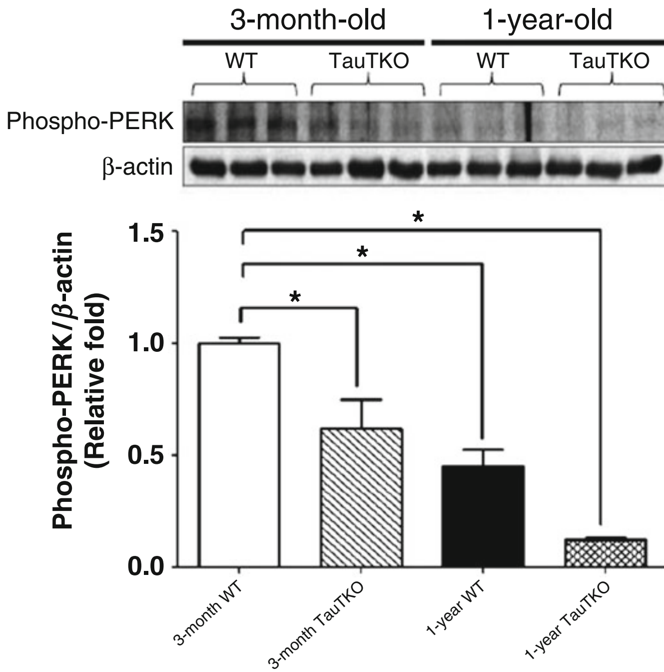

Fig. 3 Taurine depletion decreases activation of PERK. Total lysates obtained from 3 month old to 12 month old wildtype (WT) and TauTKO hearts were subjected to Western blot analysis of phosphorylated PERK. Shown in the upper panel are representative gels of phosphorylated PERK and β-actin. Values shown in the lower panel represent means ± S.E.M. of 6–9 hearts. The asterisks denote a significant difference between the comparisons shown in the figure ($p < 0.05$)

deficiency also suppresses UPR and the PERK-mediated cascade. As seen in Fig. 3, TauTKO hearts contain reduced levels of phospho-PERK. This finding is consistent with the observed acceleration in aging and in apoptosis in the TauTKO heart.

4.2 *Inflammation-Mediated Aging in TauTKO*

The proposed scheme for inflammation-mediated aging in the taurine deficient heart is shown in Fig. 4. The major effect of taurine in the heart is to ensure normal respiratory chain function, an effect tied to the conjugation of taurine with the wobble position uridine of $tRNA^{Leu(UUR)}$. The decrease in ND6 levels reduces respiratory function and enhances the generation of superoxide anion by complex I. ROS are recognized activators of a number of signaling kinases, including IκB kinase, which elevates the production of NF-κB and mediates the subsequent synthesis of a number of pro-inflammatory agents. This sequence of events leads to chronic inflammation and premature aging.

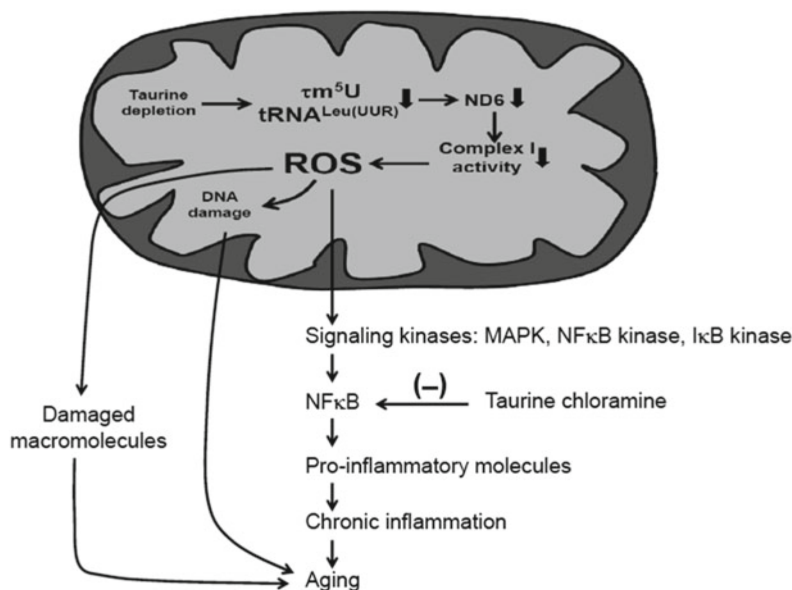

Fig. 4 Scheme for inflammation-mediated aging in taurine depleted heart. Taurine depletion reduces the levels of 5-taurinomethyluridine-tRNA$^{Leu(UUR)}$ (τm^5U-tRNA$^{Leu(UUR)}$), which in turn reduces the biosynthetic rate for the respiratory chain subunit, ND6. The activity of complex I activity declines but ROS generation increases, which leads to oxidative DNA damage and premature aging. Excessive mitochondrial ROS generation also causes the accumulation of oxidatively damaged macromolecules, another cause of premature aging. The third pathway initiated by mitochondrial generated ROS is the activation of signaling kinases, which are upstream from NF-κB. The increase in NF-κB signaling leads to the upregulation of pro-inflammatory molecules that trigger chronic inflammation and then premature aging

Taurine also functions as an anti-inflammatory agent through at least two mechanisms. First, taurine is a scavenger of HOCl, which can damage macromolecules and contribute to the severity of the inflammatory insult. Second, the product of myeloperoxide-catalyzed reaction between taurine and HOCl, taurine chloramine, also exerts anti-inflammatory actions. These include a reduction in NF-κB levels and a reduction in downstream pro-inflammatory mediators (Marcinkiewicz et al. 1998; Kontny et al. 2000).

5 Conclusions

Does taurine prolong lifespan by improving heart function? The answer to that question is a resounding yes. Taurine plays a fundamental role in the heart, but not only in the heart but in all cells, as it ensure normal function of the mitochondria, including normal production of ATP. It also regulates the production of superoxide, which

is involved in oxidative stress as well as in cell signaling and vascular relaxation. Excessive generation of ROS by the mitochondria of the taurine deficient heart contributes to both premature aging and development of a cardiomyopathy, both of which are determinants of lifespan.

The initial event in the development of the taurine deficient cardiomyopathy is mitochondrial dysfunction. However, the development of heart failure itself involves some of the same pathways of ventricular remodeling as seen in other models of heart failure. Therefore, it is not surprising that sarcoplasmic reticular Ca^{2+} mishandling plays a key role in the severity of the cardiomyopathy.

Premature aging is also an important characteristic of the taurine deficient heart. Both the accumulation of damaged macromolecules and the activation of the NF-κB pathway of chronic inflammation are involved in the acceleration of the aging process. However, taurine exerts a special influence on this pathway, as taurine chloramine decreases NF-κB and downstream pro-inflammatory mediators.

A key question is whether taurine feeding affects the aging process and the development of the cardiomyopathy. Based on the lifetime work of Dr. Junichi Azuma, it would appear that taurine therapy diminishes the severity of congestive heart failure. Clearly further studies are required to clarify the effect of taurine treatment on the aging process.

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Taurine Depletion Decreases GRP78 Expression and Downregulates Perk-Dependent Activation of the Unfolded Protein Response

Chian Ju Jong, Takashi Ito, Junichi Azuma, and Stephen Schaffer

1 Introduction

The ER is the main site for the synthesis of transmembrane, secretory and ER-resident proteins. This process is regulated by various ER chaperones and catalysts to ensure proper protein folding. Only properly folded proteins are transported to their targeted destinations, while misfolded or unfolded proteins are degraded via the ubiquitin-proteasome system (Malhotra and Kaufman 2007). However, when misfolded or unfolded proteins accumulate in the ER, glucose-regulated protein 78 (GRP78), which is a key ER chaperone, triggers the activation of the UPR (Malhotra and Kaufman 2007). UPR serves as an adaptive response to ER stress by either activating pro-survival or pro-death signaling pathways (Jager et al. 2012). Among the three transmembrane sensor proteins that participate in the UPR, protein kinase R-like ER kinase (PERK) mediates a signaling cascade that induces the translational genes associated with anti-oxidative stress responses and apoptosis (Minamino and Kitakaze 2010).

Under control conditions, PERK binds tightly to GRP78. The accumulation of misfolded or unfolded proteins triggers the release of GRP78, which activates PERK via dimerization and autophosphorylation events. As a protein kinase, activated PERK phosphorylates eukaryotic translation initiation factor 2 (eIF2α) and attenuates the

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initiation of general protein translation, thereby reducing protein synthesis (Harding et al. 2000a). However, the phosphorylation of eIF2α is essential for the translation of mRNA ATF4, a transcription factor that regulates the transcription of many genes, including those that participate in the antioxidant stress responses and in apoptosis (Back et al. 2009; Harding et al. 2003).

Recent studies suggest that oxidative stress can aggravate ER stress (Haynes et al. 2004; Malhotra et al. 2008), an effect prevented by antioxidants (Malhotra et al. 2008; Pan et al. 2012; Lin et al. 2013). Although most studies have attributed oxidative stress to excessive protein folding in the ER (Tu and Weissman 2004; Tyo et al. 2012; Dolai et al. 2011), it is also possible that damaged mitochondria produce excessive reactive oxygen species that leak into the ER and cause either an impairment in protein folding or the oxidation of several key ER chaperones (Lee et al. 2010; Yuzefovych et al. 2013). Regardless of the source of oxidative stress, ER stress often causes an elevation of GRP78 and leads to subsequent UPR activation (Jager et al. 2012; Malhotra and Kaufman 2007). However, a reduction in GRP78 levels has been observed in aging and degenerative disorders (Katayama et al. 1999; Nuss et al. 2008; Paz Gavilan et al. 2006). As GRP78 is the key regulator of UPR, it is not surprising that the PERK-eIF2α-ATF4 signaling pathway is impaired in aging animals (Hussain and Ramaiah 2007; Li and Miller 2014). A consequent of this defect is enhanced apoptosis, most likely via caspase 12 activation (Naidoo et al. 2008).

Taurine is a β-amino acid and is often associated with the prevention of oxidative stress (Gurer et al. 2001; Oliveira et al. 2010; Schaffer et al. 2009). Previously, we have shown that taurine depletion mediated by β-alanine causes mitochondrial dysfunction and oxidative stress (Jong et al. 2011a, b). As oxidative stress is generally associated with altered ER homeostasis and serves as a trigger of ER stress, we examined whether taurine depletion, like aging, decreases GRP78 levels and diminishes flux though the PERK signaling pathway. We tested this hypothesis utilizing hearts of transgenic mice lacking the taurine transporter (TauTKO).

2 Methods

2.1 Mice

Wild-type (WT) and homozygous TauTKO mice were produced by breeding heterogenous TauTKO$^{+/-}$ C57BL/6 mice. This study was conducted using 1-year-old mice. Animal handling and experimental procedures followed the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of the University of South Alabama.

2.2 Western Blot Analysis

Total lysates were prepared by homogenizing hearts in radio-immunoprecipitation assay (RIPA) lysis buffer (50 mM Tris Base, pH 8.0, 150 mM NaCl, 0.5 % deoxycholic acid, 1 % NP-40, 0.1 % sodium dodecyl sulfate). Homogenates were centrifuged at 10,000 g for 10 min and supernatants were collected as total lysates. Protein concentration was measured by the bicinchoninic assay (BCA). Protein (20–30 μg) was mixed with an equal volume of 5× sample buffer (1.25 mM Tris HCl, pH 6.8, 1 % sodium dodecyl sulfate, 10 % glycerol, 5 % β-mercaptoethanol) and then boiled for 5 min. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. The membranes then were blocked in blocking buffer (5 % milk in tris-buffered saline with Tween 20) and incubated with an appropriate primary antibody overnight at 4 °C. Membranes were washed before incubating with an appropriate secondary antibody. After washing, western blots were analyzed by enhanced chemiluminescent reagents.

2.3 Statistical Analysis

All results were reported as means ± S.E.M. The statistical significance of the data was determined using the student's *t*-test for comparison within groups. Values on $p < 0.05$ were considered statistically significant.

3 Results

The relative levels of GRP8, a key ER chaperone, are decreased in taurine-depleted hearts (Fig. 1).

In response to ER stress, GRP78 activates the PERK-eIF2α-ATF4 pathway, one of the UPR signaling cascades that regulates the transcription of anti-oxidative stress and apoptotic response genes. However, downregulation of this pathway was observed in degenerative diseases, including aging, in which GRP78 levels are reduced (Hussain and Ramaiah 2007; Katayama et al. 1999; Li and Miller 2014; Nuss et al. 2008). Similarly, in taurine-depleted hearts, activation of the PERK-eIF2α-ATF4 pathway is suppressed. Taurine depletion mediates a 30 % decrease in both the protein levels of phosphorylated and total PERK (Fig. 2). While total eIF2α was not significantly affected by taurine depletion, there was a 25 % decrease in phosphorylated levels of eIF2α (Fig. 3). The protein levels of ATF4 in taurine-depleted hearts were also significantly reduced to a level 50 % that of the WT hearts (Fig. 4).

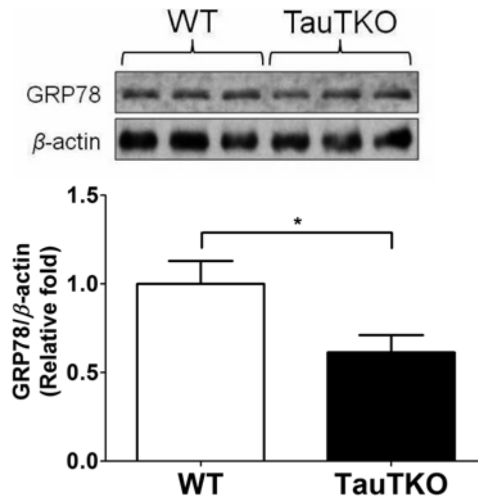

Fig. 1 Taurine depletion decreases GRP78 levels. Total lysates obtained from wild-type (WT) and TauTKO hearts were subjected to Western blot analysis of GRP78. Shown in the *upper panel* are representative gels of GRP78 and β-actin. Values shown in the *lower panel* represent means ± S.E.M. of the GRP78/β-actin ratio of 6–9 hearts. Values are expressed relative to WT, where WT is fixed at 1.0. The *asterisk* denotes a significant difference between the WT and TauTKO groups ($p < 0.05$)

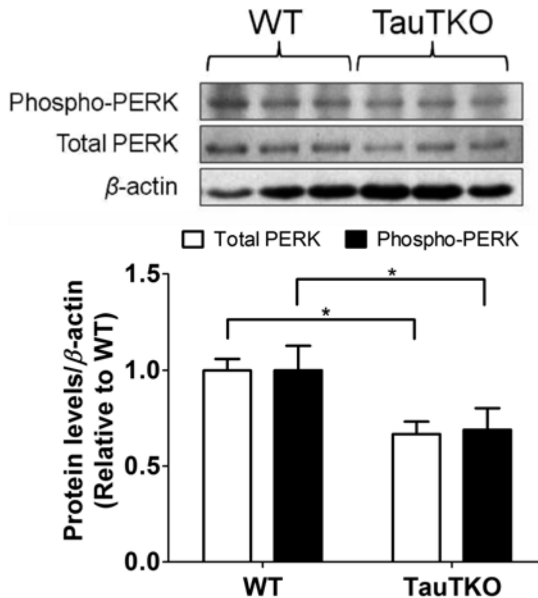

Fig. 2 Taurine depletion decreases activation of PERK. Total lysates obtained from WT and TauTKO hearts were subjected to Western blot analysis of total and phosphorylated PERK. Shown in the *upper panel* are representative gels of total PERK, phosphorylated PERK and β-actin. Values shown in the lower panel represent means ± S.E.M. of the total PERK/β-actin and phosphorylated PERK/β-actin ratios of 6–9 hearts. Values are expressed relative to WT, where WT is fixed at 1.0. Asterisks denote a significant difference between the WT and TauTKO groups ($p < 0.05$)

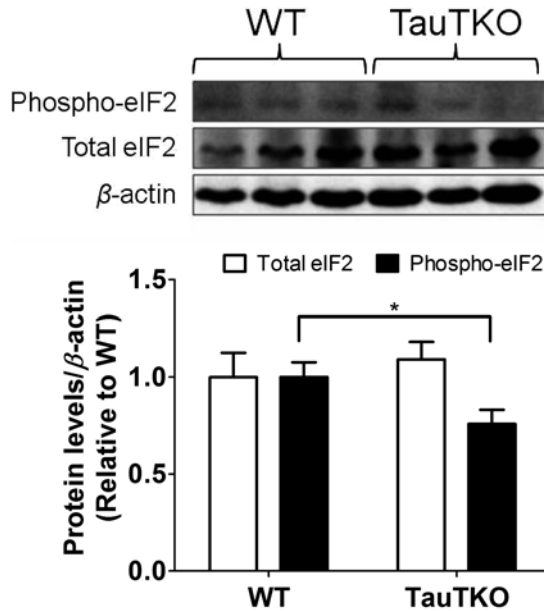

Fig. 3 Taurine depletion decreases activation of eIF2α. Total lysates obtained from WT and TauTKO hearts were subjected to Western blot analysis of total and phosphorylated eIF2α. Shown in the *upper panel* are representative gels of total eIF2α, phosphorylated eIF2α and β-actin. Values shown in the *lower panel* represent means ± S.E.M. of the total eIF2α/β-actin and phosphorylated eIF2α/β-actin ratios of 6–9 hearts. Values are expressed relative to WT, where WT is fixed at 1.0. The *asterisk* denotes a significant difference between the WT and TauTKO groups ($p < 0.05$)

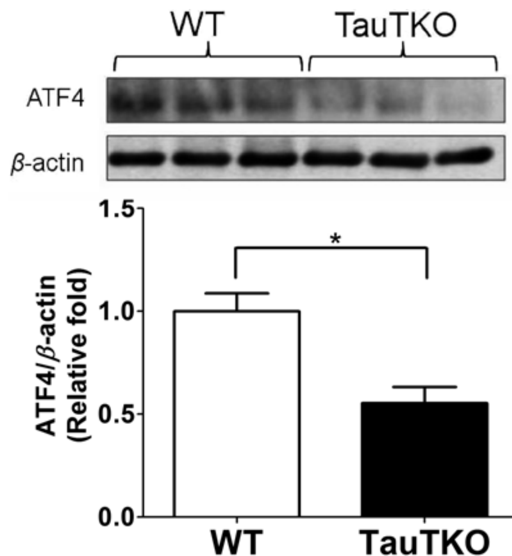

Fig. 4 Taurine depletion decreases ATF4 levels. Total lysates obtained from WT and TauTKO hearts were subjected to Western blot analysis of ATF4. Shown in the *upper panel* are representative gels of ATF4 and β-actin. Values shown in the *lower panel* represent means ± S.E.M. of the ATF4/β-actin ratio of 6–9 hearts. Values are expressed relative to WT, where WT is fixed at 1.0. The *asterisk* denotes a significant difference between the WT and TauTKO groups ($p < 0.05$)

4 Discussion

This study demonstrates that taurine depletion decreases GRP78 levels and suppresses the activation of the PERK-eIF2α-ATF4 pathway, one of the UPR signaling cascades that is essential for regulating the transcription of stress response genes.

Taurine, which is found abundantly in the heart, is particularly known for its antioxidant activity, with recent reports suggesting a relation to reduced oxidant production by the mitochondria (Chen et al. 2012; Jong et al. 2011a). The ER has an oxidizing environment rendering it susceptible to oxidative damage (Tu and Weissman 2004; Malhotra et al. 2008). Indeed, several studies have provided evidence of increased protein oxidation of several ER chaperones including GRP78 in aged tissues that are well-characterized by increased oxidative stress (Rabek et al. 2003; Nuss et al. 2008). In the present study, we showed that taurine depletion decreases GRP78 levels. As we have previously shown, taurine depletion mediated by β-alanine causes mitochondrial dysfunction and oxidative stress (Jong et al. 2011a, b). It is reasonable to expect that a link exists between mitochondrial oxidative stress and decreased GRP78 levels in response to taurine deficiency, although this hypothesis requires further investigation.

GRP78, which is the key ER chaperone responsive to ER stress, binds to several ER membrane sensor proteins (PERK, eIF2α, IREα). The activated ER membrane sensor proteins initiate UPR signaling cascades as a protective response against ER stress. On the other hand, when GRP78 is released from the ER membrane sensor proteins, it is free to bind to misfolded or unfolded proteins, thereby targeting them for degradation by the ubiquitin-proteasome system. The PERK-eIF2α-ATF4 pathway is often activated as an early event during ER stress and plays a key role in regulating the transcription of stress response genes (Back et al. 2009; Harding et al. 2000b). When activated, PERK, a serine-threonine kinase, phosphorylates eIF2α, which not only reduces global protein synthesis but also enhances mRNA translation of several key proteins that mediate cell survival, including ATF4. ATF4 is a transcription factor that enhances the transcription of genes involved in redox reactions, amino acid metabolism and stress responses (Back et al. 2009). Thus, inhibition of global protein synthesis and induction of ATF4-targeted genes syntheses help alleviate ER stress. Indeed, several pathological conditions that cause ER stress, as demonstrated by elevated levels of GRP78 and the upregulation of the PERK-eIF2α-ATF4 pathway, serve as pro-survival factors (Rutkowski et al. 2006; Saito et al. 2011). However, when ER stress is not relieved, activation of the PERK-eIF2α-ATF4 pathway can cause deleterious effects by activating C/EBP homologous protein (CHOP), a pro-apoptotic transcription factor, which mediates cell death (Rutkowski et al. 2006).

In contrast, a decrease in GRP78 expression downregulates the PERK-eIF2α-ATF4 pathway, a phenomenon observed in degenerative diseases, including aging. As GRP78 is the key ER chaperone that regulates the UPR, it is not surprising that a decline in GRP78 levels downregulates the UPR and results in the accumulation of misfolded or unfolded proteins as observed in aged tissues. A similar downregulation of the PERK-dependent pathway of the UPR is also observed in the present

study in response to taurine deficiency. While the underlying mechanism of taurine depletion-mediated downregulation of the PERK-eIF2α-ATF4 pathway is not clear, taurine supplementation has been shown to protect against ER stress (Nonaka et al. 2001; Pan et al. 2010; Yang et al. 2013). One possible explanation may be attributed to the antioxidant activity of taurine, which diminishes damage to proteins. Therefore, the present findings suggest a regulatory role for taurine in preventing ER stress.

As aging is characterized by decreased GRP78 expression and reduced activation of the UPR, our data also suggest that aging and taurine depletion are related. A characteristic of aging is the accumulation of protein aggregates that lead to impaired cellular function. This feature, however, is rarely observed in young, unstressed cells due to an efficient cellular quality control mechanism that is regulated in part by the ER (Araki and Nagata 2011; Salminen and Kaamiranta 2010). However, in aging, this quality control mechanism becomes inefficient as ER chaperones are less responsive to ER stress, as evidenced by decreased levels and activities of ER chaperones in aged tissue (Nuss et al. 2008; Katayama et al. 1999). This defect has been attributed to increased oxidation of several key ER chaperones (Rabek et al. 2003), which would agree with the mitochondrial free radical theory of aging (Cadenas and Davies 2000). This theory proposes a positive correlation between aging-related mitochondrial free radical generation and increased oxidative damage. Increased oxidative stress in aging is also associated with impaired mitochondrial function (Navarro and Boveris 2007; Zuin et al. 2008). Indeed, a decline in respiration slows electron flux along the respiratory chain and promotes the diversion of electrons to oxygen to form superoxide. Excessive superoxide generation overwhelms cellular antioxidants, thereby causing oxidative stress. Oxidative stress in turn causes cellular damage and results in more reactive oxygen species being produced; a vicious cycle that contributes to a progressive decline in cellular function. Our current findings in the taurine-depleted hearts show a close resemblance to accelerated aging. Post-mitotic tissues, such as hearts, require an efficient cellular quality control mechanism, as cardiac myocytes do not proliferate and are prone to the accumulation of protein aggregates (Groenendyk et al. 2010). As a decline in the ER stress response is associated with aging-related accumulation of oxidation-mediated protein aggregates, the present study suggests that taurine deficiency is associated with accelerated aging. The role of taurine as an anti-aging agent, however, has not been clearly established. Therefore, a thorough investigation of the aging effects of taurine deficiency is deemed necessary.

5 Conclusion

In summary, this study shows that taurine deficiency decreases GRP78 levels and downregulates the PERK-eIF2α-ATF4 pathway of the UPR. As aging is associated with a decline in ER stress response, this study also suggests that premature aging is mediated by taurine deficiency.

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Role of ROS Production and Turnover in the Antioxidant Activity of Taurine

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Abbreviations

CAT	Catalase
DHLA	Dihydrolipoic acid
ETC	Electron transport chain
G6P	Glucose-6-phosphate
G6-PD	Glucose-6-phosphate dehydrogenase
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSG	Glutathione disulfide
GST	Glutathione-S-transferase
LA	α-Lipoic acid
NNT	Nicotinamide nucleotide transydrogenase
PPP	Pentose phosphate pathway

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Prx3	Peroxiredoxin-3
RNS	Reactive nitrogen secies
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SR	Sarcoplasmic reticulum
Trx	Thioredoxin
TrxR	Thioredoxin reductase

1 Introduction

Taurine is a ubiquitous, semi-essential beta-amino acid found in very high concentration in the heart. One of its most widely recognized functions is its antioxidant activity (Pasantes-Morales and Cruz 1985; Zugno et al. 2007; Parvez et al. 2008; Cassol et al. 2010; Roy and Sill 2012). However, taurine does not function as a classical free radical scavenger, as its sulfur exists in a completely reduced state (sulfonic acid) and therefore is incapable of accepting more electrons (Arouma et al. 1988). Nonetheless, the beta-amino acid still is capable of functioning as an indirect antioxidant, either by diminishing the production of oxidants or by increasing the levels of the antioxidant defense system, which includes glutathione-S-transferase (GST), glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) (Tabassum et al. 2006; Das et al. 2010; Sevin et al. 2013; Taziki et al. 2013).

The antioxidant activity of taurine in the heart has been an area of considerable interest. The heart is dependent upon oxidative metabolism to generate ATP for muscle contraction. While the heart contains an abundant number of mitochondria for ATP generation, one untoward consequence of aerobic metabolism is the risk of excessive mitochondrial superoxide production, in which case the production of superoxide by the mitochondrial ETC exceeds the scavenging to reactive oxygen species (ROS) by the antioxidant defense enzymes, whose activity are generally low in the heart. In contrast to other members of the antioxidant defense system, the levels of the antioxidant, taurine, in the heart are extremely high, ranging from 3 mmol/kg in cow to 40 mmol/kg in mouse; levels in man are about 5 mmol/kg (Kocsis et al. 1976). These high intracellular levels of taurine require the accumulation of the amino acid from the blood. Inhibiting or knocking out the taurine transporter prevents both dietary and hepatically synthesized taurine from reaching the heart, causing intracellular levels of taurine to plunge and the heart to develop a cardiomyopathy (Novotny et al. 1991; Pion et al. 1992; Ito et al. 2008). It has been hypothesized that oxidative stress is responsible for the development of the taurine deficient cardiomyopathy. However, the mechanism underlying the antioxidant activity of taurine is an area of active research. The present review discusses the role of taurine in the regulation of oxidant production and turnover, as well as the effect of its antioxidant activity on contractile function.

2 Identity and Sources of Oxidants

Molecular oxygen is capable of accepting one, two or three electrons, forming in the process superoxide anion, hydrogen peroxide or hydroxyl radical, respectively. Superoxide is a weak oxidant but serves as a precursor of three highly reactive oxidants. In the presence of nitric oxide, superoxide is rapidly converted to peroxynitrite, a highly reactive oxidant belonging to a group of compounds referred to as reactive nitrogen species (RNS). Although the reaction between superoxide anion and nitric oxide is nonenzymatic, it is chemically favored, as it involves the reaction of the unpaired electron of nitric oxide with the unpaired electron of superoxide anion to form a product with paired electrons. Because of the limited availability of superoxide, the nonenzymatic peroxynitrite reaction competes with the superoxide dismutase-catalyzed formation of hydrogen peroxide, for superoxide anion. In the presence of modest to high levels of nitric oxide, the formation of peroxynitrite is favored over the dismutation of SOD. However, the conversion of superoxide anion to hydrogen peroxide proceeds when levels nitric oxide levels are either low or absent.

Hydrogen peroxide is a moderate oxidant capable of oxidizing electrophiles. In the presence of Fe^{2+}, superoxide is converted to a highly reactive oxidant, hydroxyl radical, a reaction that leads to the oxidation of Fe^{2+} to Fe^{3+}. This reaction, referred to as the Haber-Weiss reaction, is important in the heart because Fe^{2+} is prevalent, not only as free Fe^{2+}, but also in association with heme-containing proteins, such as myoglobin, cytochromes and oxidases/peroxidases. The t½ for hydoxyl radical in the cell is ~1 ns, indicating that it highly reactive, capable of readily transferring its unpaired electron to available electrophiles in its vicinity.

The heart is an anaerobic tissue that depends upon the availability of large amounts of ATP to drive contraction. In accordance with its ATP requirement, the major source of ROS in the heart is the mitochondrial electron transport chain (ETC), which supplies ATP for myocardial contraction. However, the ETC is also the major source of superoxide, which is formed when electrons divert from complexes I and III to the acceptor, oxygen. In normal mitochondria, electrons that enter the ETC at complexes I and II are passed on to ubiquinone which in turn passes the electrons on to complexes III and IV, the latter catalyzing the reduction of oxygen to water. This flow of electrons is closely coupled to the formation of a proton gradient, which is ultimately used to drive the biosynthesis of ATP. However, if flux of electrons through the ETC slows, electrons can be diverted away from the ETC to form superoxide anion. Conditions that lead to impaired ETC flux and the generation of superoxide by the ETC include ETC damage, impaired biosynthesis of ETC subunits, enhanced oxygen availability or excessive production of reducing equivalents.

Another major source of ROS in the heart is NADPH oxidase, an enzyme consisting of a central membrane-associated core surrounded by several cytosolic regulatory subunits (Nox1, -3, -4, -5, Duox1, -2, p47phox and p67phox). The classical isoform of NADPH oxidase is found in the neutrophil, where the enzyme serves as a source of ROS to attack foreign bodies. In the heart, NADPH oxidase initiates several signaling pathways in response to cellular stress (Jiang et al. 2011). Among the

targets of NADPH oxidase are apoptosis signal-regulating kinase-1, MAP kinase phosphatases, Akt and protein tyrosine phosphatases, all of which play a role in cell survival and cellular hypertrophy. Knocking out specific regulatory subunits of NADPH oxidase diminishes stress-induced responses, including cardiomyocyte hypertrophy, contractile dysfunction, ventricular remodeling and mortality in models of heart failure (Grieve et al. 2006; Doerries et al. 2007; Looi et al. 2008). Interestingly, two of the central neurohumoral factors, norepinephrine and angiotensin II, involved in the development of congestive heart failure, activate NADPH oxidase through a G-protein coupled pathway. Drugs that inhibit the actions of those two neurohumoral factors presently serve as the mainstay for the treatment of congestive heart failure.

The generation of ROS by xanthine oxidase has also been implicated in ischemia-reperfusion injury (Chambers et al. 1985). During ischemia, ATP is broken down to adenosine, which is subsequently converted to xanthine by the actions of adenosine deaminase, purine nucleotide phosphorylase and xanthine oxidase. The accumulation of xanthine by the ischemic heart results in the generation of ROS, as the conversion of xanthine to uric acid by xanthine oxidase results in the production of superoxide and hydrogen peroxide. Some investigators report that inhibition of xanthine oxidase protects the heart against ischemia-reperfusion injury (Chambers et al. 1985).

Inflammation is another major source of myocardial oxidative stress, as neutrophils are recruited to the heart in response to an ischemia/reperfusion insult, congestive heart failure, infective endocarditis and rheumatic heart disease. When activated during phagocytosis, neutrophils undergo a respiratory burst that leads to the generation of superoxide and other ROS, including hydrogen peroxide, hypochlorous acid, hydroxyl radical and single oxygen. These ROS are part of the armamentarium the neutrophil uses to protect the host against bacteria and other foreign invaders. However, in the diseased heart, the inflammatory response can damage normal tissue.

3 Effect of Taurine on ROS Production

The primary mechanism underlying the antioxidant activity of taurine appears to be linked to a conjugation reaction between taurine and the wobble uridine of $tRNA^{Leu(UUR)}$ forming the product 5-taurinomethyluridine-$tRNA^{Leu(UUR)}$. This post-translational conjugation reaction is important because it substantially strengthens the interaction between the UUG codon of leucine mRNA and the AAU anticodon of $tRNA^{Leu(UUR)}$. Thus, the decoding of UUG in the presence of conjugation-free $tRNA^{Leu(UUR)}$ is severely diminished, resulting in reduced expression of UUG dependent mitochondria encoded proteins. The most UUG dependent proteins are subunits of ETC complex I, therefore, in the taurine deficient heart the activity of complex I is dramatically reduced. The resulting decrease in ETC flux renders the mitochondria susceptible to superoxide production, as electrons are diverted away from complex I to the acceptor oxygen, forming superoxide anion. Recently, we found that taurine deficient cardiomyocytes and fibroblasts are oxidatively stressed, an effect reversed by taurine treatment (Jong et al. 2012). These data support the

view that taurine acts as an antioxidant, primarily by reducing the generation of superoxide by the ETC.

Li et al. (2009) have argued that the antioxidant activity of taurine also extends to NADPH oxidase, an important source of cytosolic ROS in the cardiomyocyte. They found addition of norepinephrine to the incubation medium of adult cardiomyocytes leads to cellular apoptosis, an effect they attributed to NADPH oxidase-mediated activation of calpain. Taurine treatment not only inhibited the activation of NADPH oxidase but also ROS-mediated activation of calpain and apoptosis. In their study, NADPH oxidase activity was defined as diphenyleneiodonium-sensitive activity. However, diphenyleneiodonium not only inhibits NADPH oxidase but also NADH ubiquinone oxidoreductase, an enzyme involved in the generation of superoxide by complex I of the mitochondrial ETC (Li and Trush 1998). Because taurine improves the status of complex I, it is logical to assume that the mitochondrial actions of taurine contribute to the reported inhibition of NADPH oxidase and norepinephrine-mediated apoptosis. Miao et al. (2013) also reported that taurine diminishes the expression of NADPH oxidase subunits in bacterial-induced mastitis. However, in the presence of an inflammatory response, taurine is capable of diminishing ROS production through the formation of taurine chloramine, which inhibits both the inflammatory response and NADPH oxidase activity through a reduction in the phosphorylation of p47phox and its association with the core subunits of NADPH oxidase. It is important to recognize that taurine might also influence NADPH oxidase activity by modulating NADPH content. It has been shown that taurine deficiency leads to an increase in reducing equivalents, which elevates the NADH/NAD^+ and NADPH/$NADP^+$ ratios (Mozaffari et al. 1986). Taurine deficiency also decreases glucose-6-phosphate (G6P) content, which is a substrate for G6P dehydrogenase (G6-PD), a major source of NADPH in the heart (Mozaffari et al. 1986). Thus, more work is warranted to investigate a possible link between NADPH oxidase deactivation and taurine exposure.

Oxidative stress is a major cause of injury in the ischemic-reperfused heart (Murphy and Steenbergen 2008). During the ischemia phase of an ischemia-reperfusion insult, ROS contributes to oxidative damage within the mitochondria that renders the heart susceptible to a burst of ROS generation upon reperfusion. Ueno et al. (2007) found that taurine treatment protects the heart against reperfusion injury, which appears to be largely caused by the generation of superoxide by the mitochondria. By minimizing damage to the ETC, taurine should protect the heart against excessive mitochondrial ROS generation (Schaffer et al. 2014). However, there is also evidence that the generation of superoxide by xanthine oxidase also contributes to oxidative damage during reperfusion (Chambers et al. 1985). According to Das and Sil (2012), taurine is capable of reducing xanthine oxidase activity in diabetic kidney. Although the mechanism underlying the reduction in xanthine oxidase activity was not examined, it is possible that taurine, by reducing mitochondrial ROS generation, minimizes oxidative activation of xanthine oxidase and the production of more ROS.

One of the most important functions of taurine is the neutralization of hypochlorous acid and the formation of taurine chloramine, which serves as an important anti-inflammatory agent (Marcinkiewicz and Kontny 2014). These reactions play a central role in the antioxidant activity of taurine.

4 Identity and Sources of Antioxidant Defense System

SOD, an enzyme that catalyzes the conversion of superoxide to hydrogen peroxide, exists as several isoforms. In humans, there are three isoforms; CuZnSOD which is present in the cytosol, Mn-SOD which is mitochondrial and contains manganese at the active site, and extracellular SOD (EC-SOD) (Valko et al. 2006). Mn-SOD is one of the most effective antioxidant enzymes, capable of eliminating superoxide anion in the matrix or on the inner side of the inner mitochondrial membrane (Turrens 2003). Surai et al. (1999) investigated the antioxidant profile of various tissues in newly hatched chick and found that the heart contains higher Mn-SOD activity than other tissues. The SOD content of neonates is lower than that of adults and can be further reduced by hypoxia although glucose treatment restores activity to nearly control levels in the presence of hypoxia (Anju et al. 2009). On the other hand, treatment with 100 % oxygen following a hypoxic insult causes a further increase in ROS levels, an effect associated with the induction of SOD. The combination of glucose, epinephrine and oxygen also decreases SOD activity.

CAT catalyzes the conversion of hydrogen peroxide to water. It is a membrane bound enzyme found in the peroxisomes and in heart mitochondria but not in the mitochondria of other tissues (Dhalla et al. 2000; Turrens 2003). Although the activity of CAT is low in the myocardium, it plays an important role in protecting the heart from ischemia-reperfusion insults (Dhalla et al. 2000). Because the active site of CAT binds two moles of hydrogen peroxide, the reaction does not proceed at low levels of hydrogen peroxide. The enzyme is phosphorylated and stimulated by non-receptor tyrosine kinases, c-Abl and Arg, which increase CAT activity at low hydrogen peroxide concentrations (Rhee et al. 2005). It has also been shown that CAT activity is suppressed under hypoxic conditions in the neonatal rat heart. This suppression is ameliorated by glucose supplementation, but if oxygen and epinephrine treatment is combined, the ameliorative effect of glucose is abolished, an effect analogous to that of SOD (Anju et al. 2009).

Vitamin C (ascorbic acid) is a powerful non-enzymatic antioxidant that protects membranes against oxidation. It functions in an aqueous environment, such as in lungs and lens. Almost 99.9 % of vitamin C exists as $AscH^-$, which can react with free radicals to produce semidihydroascorbate radical, which is surprisingly rather nonreactive (Valko et al. 2006).

Vitamin E, which is present in eight different forms, functions as an antioxidant in hydrophobic environments. The major membrane-bound antioxidant in humans is α-tocopherol, which is highly effective against the process of lipid peroxidation. A synergistic reaction involving vitamin C and α-tocopherol results in the oxidation of α-tocopherol to an α-tocopherol radical and the reduction of ascorbic acid (Valko et al. 2006). The α-tocopherol radical and the reduced form of ascorbic acid can also couple with α-lipoic acid and glutathione (Fig. 1). Both vitamins C and E are found in the cytoplasm of the heart. Although there is evidence that vitamin E protects the ischemic heart, some epidemiological data have yielded contradictory results, revealing the need of further experimentation in human subjects.

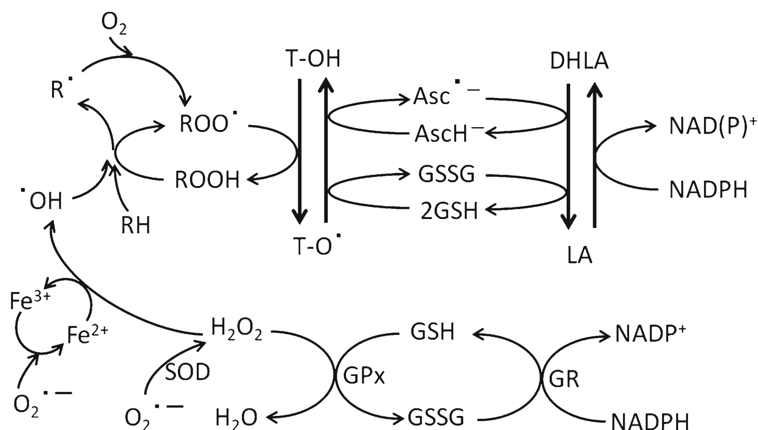

Fig. 1 Major antioxidant defense mechanisms. Superoxide anion ($O_2^{\cdot -}$) is converted to hydrogen peroxide (H_2O_2) by the enzyme superoxide dismutase (SOD). H_2O_2 in turn is converted to H_2O by glutathione reductase (GPx) in a reaction that is coupled to the conversion of reduced glutathione (GSH) to oxidized glutathione (GSSG). Glutathione reductase (GR) reduces GSSG to GSH using NADPH as the reducing agent. Alternatively, H_2O_2 can be reduced to hydroxyl radical ($OH^{\cdot}$) via the Haber-Weiss reaction. Hydroxyl radical is extremely reactive and extracts unpaired electrons from a fatty acid (RH) or a lipid radical ($R^{\cdot}$), leading to the production of a lipid peroxy radical ($ROO^{\cdot}$). Vitamin E (T-OH) reduces the peroxy radical to a hydroperoxide but is converted to a vitamin E radical in the process. Active vitamin E is restored by the reduction of the radical by either GSH or ascorbic acid ($AscH^{-}$). Dihydrolipoic acid (DHLA) is capable of reducing GSSG and Asc^{*-}

Thioredoxin (Trx) is a small multifunctional, disulfide-containing protein, which is found at levels 100–10,000-fold less than those of GSH. Trx can undergo a coupled redox reaction, in which two sulfhydryl groups of Trx are converted to a disulfide unit while a disulfide bridge of a protein is reduced to two sulfhydryls. Thioredoxin reductase (TrxR) uses NADPH to catalyze the reduction of oxidized Trx to its reduced form (Valko et al. 2006). Both Trx and TrxR have isoforms; Trx1 and TrxR1 are localized to the cytosol while Trx2 and TrxR2 are found in the mitochondria. TrxR2 is a FAD-containing selenoenzyme that reduces the disulfide form of Trx2 using matrix NADPH (Hurd et al. 2005). Trx2 activity resembles that of peroxiredoxin-3 (Prx3), which is present exclusively in the mitochondria and rapidly reacts with hydrogen peroxide. In addition to detoxifying peroxynitrite, it scavenges as much as 90 % of the available hydrogen peroxide. It has been suggested that mitochondrial oxidative stress plays an important role in the development of heart failure. Hence, mitochondrial specific antioxidants reduce the risk of heart failure by scavenging damaging ROS (Marí et al. 2013; Murphy 2012; Tsutsui et al. 2009).

LA is a disulfide derivative of octanoic acid, also called thioctic acid. Because it is both water and fat-soluble, it is distributed in both cellular membranes and the cytosol. It is rapidly converted to its reduced dithiol form, dihydrolipoic acid (DHLA). Both LA and DHLA are strong antioxidants that function as scavengers of

ROS, regenerators of other antioxidants, chelators of redox metals and activators of oxidized proteins (Valko et al. 2006). The oxidation of DHLA is coupled to the reduction of oxidized glutathione (GSSG) and ascorbate, which in turn reduces α-tocopherol radical to regenerated vitamin E (Fig. 1). Recently, LA was found to attenuate diabetes-associated upregulation of p22phox and p47phox expression, leading to a reduction in NADPH-induced ROS generation. Several studies have reported that LA is cardioprotective, but further studies are required to establish its effectiveness in the heart relative to that of the other antioxidants (Ghibu et al. 2009).

Carotenoids are pigments found in plants and microorganisms. Their conjugated double-bonds play an important role in their antioxidant activity, which involves the protection of lipids against peroxidative damage (Valko et al. 2006). Lycopene, one of the most abundant dietary carotenoids, protects against congestive heart failure even at relatively low levels (Lennie et al. 2013). Vitamin A, also one of the carotenoids, is reported to significantly reduce isoproterenol-induced myocardial injury, presumably by diminishing oxidative stress and stabilizing membranes (Pipaliya and Vaghasiya 2012). β-carotene (pro-vitamin A), a carotenoid contained in human diet, fruits and colored vegetables, is present in the cytoplasm of the heart. It reportedly increases the GSH/GSSG ratio in the heart and liver of rats and to prevent collagen biosynthesis and fibrosis (Novo et al. 2013). Although many epidemiologic studies have suggested that higher plasma β-carotene content is associated with a lower risk of heart disease, several clinical trials have claimed that β-carotene supplementation has either no or a negative effect on the risk of heart disease (Voutilainen et al. 2006). It is likely that β-carotene consumed in the diet benefits the heart, while high levels of β-carotene used in clinical trials might have an adverse effect.

Polyphenols are classified into four categories, based on the number of phenolic rings and structural moieties (phenolic acids, flavonoids, stilbenes and lignans). For example, flavonoids contain a diphenylpropane moiety consisting of two aromatic rings linked through three carbons that together usually form an oxygenated heterocyclic compound. Flavonoids are commonly synthesized by plants as second metabolites and constitute the most important single group of polyphenols. They are subclassified into six groups: flavonols, flavones, isoflavones, flavanones, anthocyanidis and flavonols. The antioxidant activity of polyphenols resides in their ability to induce antioxidant enzymes, such as SOD, CAT, GST and Gpx. Polyphenols protect the heart by inhibiting ischemia/reperfusion injury, hyperlipidemia, hypertension, inflammation, atherosclerosis and age-related changes (Khurana et al. 2013; Valko et al. 2006). They also chelate trace metals, which presumably contributes to their antioxidant activity (Malireddy et al. 2012).

5 Glutathione-Linked ROS Scavenging System

GSH is a tripeptide, thiol-containing, multifunctional intracellular non-enzymatic antioxidant. It is found in fairly high concentrations throughout the cell, including the cytosol (1–11 mM), the nucleus (3–15 mM) and the mitochondria (5–11 mM).

During its reaction with other radicals, GSH is oxidized into a thiyl radical; two thiyl radicals can then dimerize into GSSG. Thus, the GSH/GSSG ratio is a good marker of oxidative stress. Although GSH is a reducing agent, GSSG can react with a protein sulfhydryl group to form a protein–glutathione-mixed disulfide.

The redox state of GSH is determined in part by nonenzymatic reactions involving proteins, ROS and vitamins. It not only serves as a scavenger of hydroxyl radicals and singlet oxygen but is also capable of regenerating vitamins C and E and ensuring the proper sulfhydryl content of proteins (Fig. 1).

Several key enzymes also alter the redox state of glutathione. One of those enzymes is GPx, which exists in two forms. One of the forms is a selenium-independent GST while the other is a selenium-dependent GPx (Valko et al. 2006). Hydrogen peroxide, which is formed during the dismutation of superoxide anion by SOD, is largely decomposed by GPx (Turrens 2003). The GPx reaction uses GSH to neutralize either hydrogen peroxide or organic peroxides (ROOH), with the former converted to water and the later to alcohol. In the process, GSH is oxidized to GSSG (Oka et al. 2012). By comparison, GST catalyzes the destruction of organic peroxides but not that of hydrogen peroxide (Turrens 2004). There are several isoforms of GPx, the major isoform being GPx1, which is localized mainly in the cytosol where it scavenges hydrogen peroxide. GPx4, which is attached to the inner mitochondrial membrane facing the matrix, readily neutralizes lipid hydroperoxides and is therefore recognized for its ability to protect membranes against oxidative damage. The mitochondria, which are a major source of ROS, contain significant levels of both GSH and GPx (Marí et al. 2013; Murphy 2012). Interestingly, the heart of newly hatched chicks contains high GPx levels. GPx is thought to contain much higher hydrogen peroxide scavenging activity compared with catalase or SOD in rat heart (Dhalla et al. 2000; Tsutsui et al. 2006). The heart from newly hatched chicks contains higher GPx dependence than other tissues (Surai et al. 1999).

Glutathione reductase (GR) couples the reduction of GSSG to the oxidation of NADPH. In the cytosol, the GR reaction is dependent upon the availability of NADPH formed by two reactions of the pentose phosphate pathway (PPP). The first step, which is rate limiting, results in the generation of NADPH as G6P is oxidized to 6-phosphogluconic acid. Because the PPP is not very active in the heart, the competition between G6-PD of the PPP and phosphohexose isomerase of the glycolytic pathway for available G6P is a major determinant of NADPH generation by the PPP. Also regulating G6-PD activity is the content of NADPH, which feedback inhibits the formation of more NADPH, and GSSG, which reverses the effects of NADPH (Zimmer 1992).

6 Antioxidant Defense System of the Heart

Mitochondria are plentiful in the heart, therefore, the heart requires an active antioxidant system to maintain the balance between ROS production, which is very active in heart, and ROS neutralization, which involves the antioxidant enzymes and the GSH redox system. The antioxidant enzymes located in the mitochondria

include Mn-SOD, CAT, GPx and GR. However, a central role in the maintenance of redox balance falls on the NADPH-dependent enzymes (GR, Trx, peroxiredoxin III, and glutaredoxin). NADPH in the mitochondria is mainly supplied by three pathways: $NADP^+$-dependent isocitrate dehydrogenase, malic enzyme and nicotinamide nucleotide transhydrogenase (NNT), with NNT responsible for more than 50 % of mitochondrial NADPH formation. NNT catalyzes the reduction of $NADP^+$ to NADPH, a half reaction coupled to the oxidation of NADH to NAD^+ (Yin et al. 2012; Garcia et al. 2010). Also important is the mitochondrial GSH:GSSG ratio, which is normally greater than 100:1. When ROS production overwhelms the antioxidant defense system, GSH is oxidized to GSSG, although there is no transporter to export GSSG out of the mitochondria. Hence, to maintain the redox state, GSSG is used in the glutathionylation of proteins. It is known that complexes I and IV, aconitase, and pyruvate dehydrogenase can undergo glutathionylation, a reaction that decreases their activities. However, glutathionylation of cardiac proteins has been implicated in several cardiovascular diseases (Pastore and Piemonte 2013).

7 Effect of Taurine on Antioxidant Defense System

Most of the studies examining the link between taurine and the antioxidant defense system have focused on the liver, which is a unique tissue because taurine is both synthesized in the liver and used by the liver to conjugate bile acids and $tRNA^{Leu(UUR)}$. Oxidative stress has been commonly associated with reductions in the levels and activities of SOD, CAT, GPx, GST, GSH, GR and G6-PD in the liver, an effect prevented by taurine treatment (Das et al. 2010; Devi and Anuradha 2010; Hagar 2004; Pushpakiran et al. 2004; Tabassum et al. 2006; Taziki et al. 2013). These findings are consistent with the view that oxidative stress damages the antioxidant enzymes. By minimizing the degree of oxidative stress, taurine prevents the decline in activity of these antioxidant enzymes.

As in the liver, taurine treatment of the oxidatively-stressed heart is associated with reversal of ROS-mediated reductions in antioxidant enzyme activity (Pushpakiran et al. 2004; Shiny et al. 2005; Sahin et al. 2011; Yang et al. 2013). In a related study, Pansani et al. (2012) showed that taurine deficiency is associated with a decrease in the activities of myocardial GPx and CAT. Although most reports administer large amounts of taurine to oxidatively-stressed animals, it is relevant that β-alanine-mediated taurine deficiency promotes the decline in GPx and CAT, suggesting that intracellular taurine levels might regulate the activity of the antioxidant defense system. However, in a study in which taurine levels were examined, there was no correlation between changes in the activity of the antioxidant enzymes and taurine levels (Anand et al. 2011). Nonetheless, the preponderance of evidence suggests that taurine treatment is capable of reducing the degree of oxidative damage to the antioxidant enzymes. Remaining to be determined is whether physiological levels of taurine modulate the activity of the antioxidant defense system. Moreover, the possibility that taurine might alter the expression of the antioxidant defense enzymes should be considered.

8 Effect of ROS and Taurine on Contractile Function

It is widely recognized that ROS diminish contractile function, a process thought to contribute to both acute cardiac injury and chronic development of heart failure. In this section, we focus on the primary mechanisms involved in ROS-mediated acute cardiac injury and contractile dysfunction. The role of ROS in the development of cardiac hypertrophy and congestive heart failure has been the topic of a recent review article by Ito et al. (2014).

Maximal force of contraction depends upon the viability of the muscle proteins, the rate of ATP biosynthesis and proper handling of Ca^{2+}. Although the most important action of taurine in the heart appears to be the maintenance of normal ETC function, moderate depletion of taurine does not alter cellular ATP levels (Mozaffari et al. 1986). However, impaired ETC function often leads to excessive mitochondrial ROS production, which can contribute to Ca^{2+} mishandling by the heart. Therefore, the present section is limited to the regulation of Ca^{2+} movement and contractile function by ROS and taurine.

The rate of cardiac contraction is normally determined by the rate at which the sinoatrial nodal pacemaker cells generate their stimulatory impulses. When these impulses reach the ventricle, they activate Na^+ channels to cause depolarization of the cardiomyocyte. The resulting change in membrane potential activates the voltage-dependent L-type Ca^{2+} channels situated on the cell membrane. Once activated, these channels remain open for a short period of time, during which Ca^{2+} enters the cell. Satoh and Sperelakis (1993) have reported that exposure of isolated chick cardiomyocytes to medium containing 10^{-7} M Ca^{2+} and 20 mM taurine inhibits Ca^{2+} transport by the L-type Ca^{2+} channel, however, they did not examine the effect of physiological concentrations of extracellular taurine (normally ~50 μM) at physiological concentrations of plasma Ca^{2+} (normally ~2.5 mM). There is also some evidence that ROS can inhibit L-type Ca^{2+} channel activity, however, taurine lacks free radical scavenging activity and would be incapable of directly reducing the levels of both hydroxyl radical and hydrogen peroxide (Zima and Blatter 2006). Thus, the L-type Ca^{2+} channel is unlikely to be a site of taurine action.

Although inhibition of the L-type Ca^{2+} channel abolishes myocardial contractile function, the amount of Ca^{2+} entering the cell via the L-type Ca^{2+} channel is insufficient to maximally stimulate contraction. Indeed, L-type current is a minor source of Ca^{2+} compared to the next step in the Ca^{2+} cycle, the release of Ca^{2+} from the intracellular Ca^{2+} storage vesicles of the sarcoplasmic reticulum (SR). The size of the SR Ca^{2+} stores ensures an important role for the SR in both normal contraction and heart failure. ROS are capable of oxidizing key cysteine residues of the SR channels involved in Ca^{2+} release, known as the Ca^{2+}-gated SR Ca^{2+} channels. Modification of these channels contributes to the development of heart failure by promoting Ca^{2+} leakage from the SR Ca^{2+} storage vesicles (Zima and Blatter 2006; Terentyev et al. 2008). Thus, the antioxidant activity of taurine might improve contractile function by preventing SR Ca^{2+} leakage although high extracellular taurine (20 mM) does not directly influence SR Ca^{2+} leakage from skinned skeletal muscle

fibers (Bakker and Berg 2002). Thus, the SR Ca^{2+} release channels (ryanodine channels) are unlikely to be major sites of taurine action.

In contrast to the ryanodine receptor, taurine has a significant influence on the SR Ca^{2+} ATPase (Steele et al. 1990; Bakker and Berg 2002). The SR vesicles are surrounded by a medium rich in taurine (5–30 mM), therefore, the effects of taurine on Ca^{2+} uptake by the SR Ca^{2+} pump are physiologically important. Steele et al. (1990) found that at a submaximal concentration of Ca^{2+} (0.12 μM) taurine exposure (over a concentration range of 0.01–40 mM) increased Ca^{2+} loading of the SR, thereby promoting an increase in caffeine-induced contracture of the skinned rat heart. On the other hand, 30 mM taurine reduced the amplitude of caffeine-induced contracture of skinned rat heart bathing in medium containing a comparatively high Ca^{2+} concentration (0.47 μM). Thus, in the hypodynamic heart exposed to low concentrations of Ca^{2+}, physiological concentrations of taurine increase contractile function by facilitating Ca^{2+} loading of the SR. However, in the Ca^{2+} overloaded heart, taurine decreases contractile function. The mechanism underlying this action of taurine has not been established. However, recently we found that taurine depletion leads to a decline in SR Ca^{2+} ATPase activity and in the phosphorylation state of phospholamban, both effects that are consistent with the actions of taurine on SR Ca^{2+} uptake. It remains to be determined if ROS contribute to the reduction in the phosphorylation state of phospholamban and in SR Ca^{2+} ATPase activity. We have found that β-alanine-mediated taurine loss is associated with prolongation of cardiomyocyte Ca^{2+} transients, delayed relaxation of the taurine deficient heart and an elevation in cardiomyocyte ROS content (Schaffer et al. 2000; Jong et al. 2012). Although it has been established that ROS inhibit SR Ca^{2+} ATPase activity (Zima and Blatter 2006), a key experiment has not been performed, namely, determining the effect of antioxidant therapy on myocardial relaxation and ROS content.

9 Conclusion

One of the most important actions of taurine is its antioxidant activity. It has been documented that taurine is not a characteristic scavenger of ROS. Instead, several indirect mechanisms contribute to its antioxidant activity, including reducing ROS production by complex I of the ETC, limiting the activation of xanthine oxidase and interfering with ROS-producing inflammatory reactions. Taurine treatment also elevates the levels of the antioxidant defense system, largely by preventing the loss of the antioxidant enzymes through oxidative damage.

The development of a taurine deficient cardiomyopathy can be traced to a decline in the antioxidant activity of the heart. Not only does the accumulation of ROS in the taurine deficient heart cause cardiomyocyte death, but it leads to impaired handling of Ca^{2+}. Consequently, both systolic and diastolic function of the heart are impaired, as SR Ca^{2+} pump activity is reduced.

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Taurine Supplementation Prevents the Adverse Effect of High Sugar Intake on Arterial Pressure Control After Cardiac Ischemia/Reperfusion in Female Rats

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Abbreviations

AST	Aspartate aminotransferase
BSHR	Baroreflex sensitivity control of heart rate
BSRA	Baroreflex sensitivity control of renal nerve activity
CG	Control with high sugar intake
CG+T	CG plus taurine supplementation
CK-MB	Creatine kinase-MB
CPR	Cardiopulmonary resuscitation
CW	Control without high sugar intake
CW+T	CW plus taurine supplementation
HF	High frequency
i.p.	Intraperitoneal
IR	Ischemia/reperfusion
LF	Low frequency
NT-proBNP	N-terminal prohormone brain natriuretic peptide
RAS	Renin-angiotensin system
SD	Sprague-Dawley
Trop-T	Troponin T

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1 Introduction

Epidemiological and experimental studies indicate that cardiac ischemia/reperfusion (IR), an episode of cardiac ischemia followed by restoration of blood flow (reperfusion), is one of the debilitating cardiovascular diseases that develop in the fetus and become overt during life (Frohlich et al. 2013; Roger et al. 2012; Yamori et al. 2010). Cardiac IR injury consists of both reversible and irreversible physiological, structural, and biochemical changes that develop during the IR insult. Sex, age, body weight, body activity, and diets are proposed to affect the outcome of cardiac IR (Sack and Murphy 2011; Yamori et al. 2010). Although high fat, low carbohydrate or low fat, high carbohydrate or high carbohydrate and fat diets have been reported to underlie hypertension (Brown et al. 2009; Yamori 2006), their effects on cardiac IR injury are still controversial (Miki et al. 2012). For adult Chinese, epidemiologic studies report a low arterial pressure and blood sugar, as well as a low incidence of cardiovascular disease in men daily consuming high carbohydrate than men and women consuming low carbohydrate (Zhu et al. 2014). This relationship is reversed for people in western countries that usually consume a high fat and carbohydrate diet (Brown et al. 2009; Yamori 2006). Further, the effect of both type 1 and type 2 diabetes mellitus on cardiac IR injury is one of the most controversial issues; i.e., some report exacerbating effects of diabetes while others observe no effect although type 1 diabetes is usually associated with little to minimal effect (Miki et al. 2012). Furthermore, the effect of diabetes on cardiac IR may not depend on only hyperglycemia. Many animal studies indicate that experimental hyperglycemia before, during, and/or after cardiac IR, in most studies fail to affect cardiac IR injury, except when plasma glucose is raised and sustained above 500 mg/dl (Miki et al. 2012).

Our previous experiment indicates that, in adult male rats, high sugar intake from weaning throughout adult life significantly increases cardiac injury and depresses baroreceptor reflex sensitivity 2–3 days after cardiac IR, despite nonfasting blood glucose averaging below 110 mg/dl (Kulthinee et al. 2010; Roysommuti et al. 2009). These effects are more pronounced in adult rats perinatally depleted of taurine followed by a high sugar diet after weaning (Kulthinee et al. 2010; Roysommuti et al. 2009). Together, these data suggest that high sugar intake has an adverse impact on ischemia/reperfusion-induced myocardial damage and arterial pressure control, particularly in animals perinatally depleted of taurine. Elevated plasma N-terminal prohormone brain natriuretic peptide (NT-proBNP) confirmed significant ventricular injury following cardiac IR in male rats (Kulthinee et al. 2010); these cardiac injury markers are released mainly from the cardiac ventricle (Bohm et al. 2011). Whether these effects differ between male and female rats has not been studied.

Taurine is an abundant β-amino acid found in very high concentration in the heart. Addition of taurine to the drinking water of rodents 6 months prior to cardiac

IR protects the heart against reactive oxygen species generation (Hanna et al. 2004). Clinical studies indicate that patients receiving a rapid intravenous infusion of 5 g of taurine before bypass surgery exhibit fewer necrotic cells and less lipid peroxidation damage after completion of the procedure than patients infused with medium lacking taurine (Milei et al. 1992). In a related study, arrested heart stored in St. Thomas's cardioplegic solution containing 10 mM taurine are more resistant to storage-induced ischemic injury than arrested hearts stored for 6 h in cold cardioplegic solution lacking taurine (Oriyanhan et al. 2005). Taurine feeding (200 mg/kg/day) diminishes elevations in oxidative stress, inflammation, and swelling during 5 h of cold isotonic storage (Sahin et al. 2011). In addition, taurine treatment at the time of reperfusion protects the ischemic heart against reperfusion injury, including contractile dysfunction, creatine kinase release, and lipid peroxidation (Sahin et al. 2011; Ueno et al. 2007). This advantage of taurine may be due to the fact that taurine possesses many activities, especially antioxidation, cell volume regulation, and inhibition of renin-angiotensin system (RAS) and sympathetic nerve activity (Roysommuti and Wyss 2014).

Increases in oxidative stress, RAS overactivity, sympathetic nerve overactivity, and cell volume dysregulation are main mechanisms causing cardiac injury and malfunction in IR (Frohlich et al. 2013; Schaffer et al. 2014b). In addition, taurine supplementation can prevent and improve sugar-induced hypertension in animal models (Feng et al. 2013; Harada et al. 2004; Nandhini and Anuradha 2004; Rahman et al. 2011). The present study tests the hypothesis that taurine supplementation ameliorates the effect of high sugar intake on cardiac injury and arterial pressure control after cardiac ischemia/reperfusion in adult female rats.

2 Methods

2.1 Animals

Sprague–Dawley (SD) rats were bred at the National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand and maintained at constant humidity (60 ± 5 %), temperature (24 ± 1°C), and light cycle (06.00–18.00 h). Pregnant SD rats were fed normal rat chow and tap water from conception to weaning. After weaning, female offspring were fed the normal rat chow and given either 5 % glucose in tap water (CG) or tap water alone (CW) throughout the study. Starting a week before surgery, half of the rats in each treatment group were supplemented with 3 % taurine in tap water (CG plus taurine supplementation, CG + T; CW plus taurine supplementation, CW + T) until the end of the experiment. All experimental procedures were approved by the Khon Kaen University Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health guidelines.

2.2 Experimental Protocol

At 7–8 weeks of age, rats were anesthetized with sodium pentobarbital (Nembutal, 50 mg/kg, i.p.). After catheterization of the femoral artery for arterial pressure measurement and blood sampling and femoral vein for fluid and drug infusions, the arterial pressure pulse was continuously monitored (BIOPAC Systems, Goleta, CA, USA). A tracheal tube was slowly and carefully inserted through the mouth. After a 15–20 min acclimation period and while still under deep anesthesia, cardiac ischemia was induced by asphyxia through direct tracheal tube clamping (Kulthinee et al. 2010). When the heart arrested and mean arterial pressure fell below 60 mm Hg, the clamp was released and cardiopulmonary resuscitation (CPR) was immediately performed by manual chest compression at 60 times/min. Ventilation was continuously controlled by a ventilator through the tracheal tube. The return of arterial pressure pulse and self-respiration indicated the success of CPR (usually within 2 min).

Two days later, arterial pressure and heart rate were continuously recorded in conscious rats before and during infusion of phenylephrine (increased arterial pressure) and sodium nitroprusside (decreased arterial pressure) to assess the baroreflex sensitivity. One-day later, rats were anesthetized with Nembutal, tracheostomized, and arterial pressure pulse recorded continuously. Body temperature was servo-controlled at 37 ± 0.5 °C by a rectal probe connected to a temperature regulator controlling an overhead heating lamp. Laparotomy was performed by longitudinal incision on the midline area of the abdomen. Then, the right renal artery was exposed and a stainless steel electrode (12 MΩ, 0.01 Taper, A-M System; Sequim, Washington, USA) was slowly inserted to the renal nerve, using a gold-plated electrode holder connected to a micromanipulator. A second electrode was attached to snipped abdominal muscle close to the recording site. Both electrodes were connected to a DAM-80 differential amplifier (World Precision Instruments, Sarasota, Florida, USA) and the BIOPAC Systems (Goleta, California, USA). After at least 15 min of rest and baseline data recording, baroreflex mediated renal nerve activity was assessed by infusion of phenylephrine or sodium nitroprusside.

After the study of baroreflex sensitivity, animals were rested for 30 min. Then, arterial blood samples were obtained and plasma components were immediately separated for measurements of sodium (Na^+), potassium (K^+), bicarbonate (HCO_3^-), chloride (Cl^-), creatinine (Cr), blood urea nitrogen (BUN), hematocrit (Hct), creatine kinase–MB (CK-MB), troponin T (Trop-T), aspartate aminotransferase (AST), and NT-proBNP (Srinagarind Hospital Laboratory Unit; Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand). Finally, all animals were sacrificed with a high dose of Nembutal and heart weights were collected.

2.3 Data Analyses

Mean arterial pressure, heart rate, power spectral density of arterial pressure pulse, and renal nerve activity were analyzed by using the Acknowledge software (BIOPAC Systems, Goleta, California, USA). Changes in renal nerve activity or heart rate per changes in arterial pressure following phenylephrine or sodium nitroprusside infusion were used to measure baroreceptor reflex control of renal nerve activity (BSRA) or heart rate (BSHR). The autonomic nervous system control of arterial pressure was estimated by analyzing the power spectral density of low (LF, 0.3–0.5 Hz; the sympathetic nerve activity dominant) and high (HF, 0.5–4.0 Hz; the parasympathetic nerve activity dominant) frequency components of arterial pressure pulse (Fourier analysis) (Roysommuti et al. 2009). The absolute values were normalized by the total power (LF + HF).

All data are expressed as means ± SEM. Statistical comparisons among groups were performed using one-way ANOVA and a *post hoc* Duncan's Multiple Range test and paired t-tests (two tails) were used to indicate significant differences among groups and within groups, respectively (Statmost version 3.5, Dataxiom Software, USA). The significant criterion was a *p*-value less than 0.05.

3 Results

At 7–8 weeks of age, body weight, heart weight (Table 1), mean arterial pressure, and heart rate (Fig. 1, Before) were not significantly different among the four groups. Three days after cardiac IR induction, all groups displayed similar values of plasma electrolytes, blood urea nitrogen, plasma creatinine level, and hematocrit (Table 1). While plasma AST, Trop-T, and NT-proBNP levels three days after cardiac IR were not significantly different among the four groups, plasma CK-MB significantly decreased in CW + T and CG + T compared to CW and CG, respectively (Table 2). Compared to control, high glucose intake since weaning did not affect cardiac injury markers.

After cardiac IR, unconscious mean arterial pressures significantly increased in all groups compared to their corresponding values before IR values; however, the after IR values were not significantly different among the four groups (Fig. 1). Although conscious mean arterial pressures were significantly higher than their corresponding unconscious values, they were not significantly different among the four groups. While cardiac IR significantly increased heart rates in CW but not CG groups, taurine supplementation significantly increased heart rates in both groups (CW + T and CG + T). Further, the increased heart rates among the CW, CW + T, and CG + T groups were not significantly different in either conscious or unconscious rats.

Table 1 Body weight (BW), heart weight (HW), plasma electrolytes (Na^+, K^+, Cl^-, HCO_3^-), blood urea nitrogen (BUN), plasma creatinine (Cr), and hematocrit (Hct) after cardiac ischemia/reperfusion

	BW (g)	HW (g)	Na^+ (mEq/l)	K^+ (mEq/l)	Cl^- (mEq/l)	HCO_3^-(mEq/l)	BUN (mg/dl)	Cr (mg/dl)	Hct (%)
CW	190±1	0.78±0.01	143±1	4.52±0.04	99.8±0.7	25.6±0.3	15.9±0.8	0.62±0.05	39.5±0.4
CW+T	191±2	0.77±0.01	144±1	4.53±0.06	98.3±0.6	25.0±0.4	14.6±1.3	0.50±0.09	38.3±0.7
CG	190±1	0.78±0.01	144±1	4.47±0.03	99.2±1.0	24.8±0.5	16.8±0.8	0.57±0.07	38.0±0.6
CG+T	188±2	0.78±0.01	144±1	4.50±0.04	98.6±1.2	24.6±3.0	14.9±1.4	0.48±0.09	38.5±0.9

Data are means±SEM ($n=6$ each group). No significant difference was observed among the groups

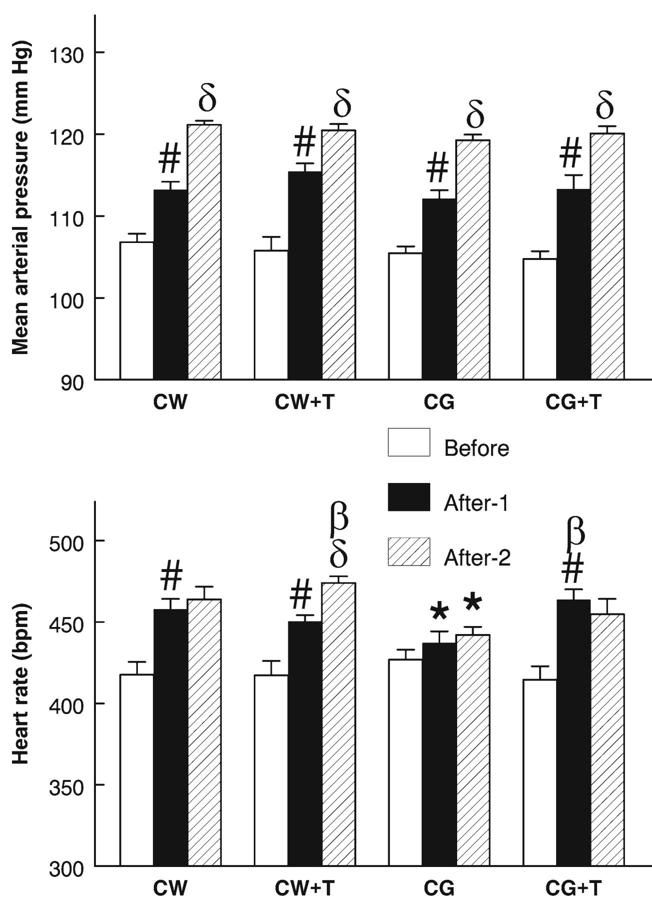

Fig. 1 Mean arterial pressure (*upper*) and heart rate (*lower*) before (unconscious) and after (After-1, unconscious; After-2, conscious) cardiac ischemia/reperfusion ($^{\#,*,\beta,\delta}$ $P<0.05$ compared to corresponding Before, CW, CG, and After-1, respectively; *CW* control without high sugar intake ($n=7$), *CW+T* CW plus taurine supplementation ($n=8$), *CG* control with high sugar intake ($n=8$), *CG+T* CG plus taurine supplementation ($n=7$))

Table 2 Plasma cardiac injury markers after cardiac ischemia/reperfusion

	AST (μg/l)	CK–MB (μg/l)	Trop-T (ng/ml)	NT-proBNP (pg/ml)
CW	157±7	299±7	<0.01	<5.00
CW+T	145±5	245±5*	<0.01	<5.00
CG	156±6	313±7	<0.01	<5.00
CG+T	140±6	250±9*	<0.01	<5.00

Data are means ± SEM ($n=6$ each group)
*$P<0.05$ compared to CW, *CW* control without high sugar intake, *CW+T* control plus taurine supplementation, *CG* control with high sugar intake, *CG+T* CG plus taurine supplementation, *AST* aspartate aminotransferase, *CK-MB* creatine kinase-MB, *Trop-T* troponin T, *NT-proBNP* N-terminal prohormone brain natriuretic peptide

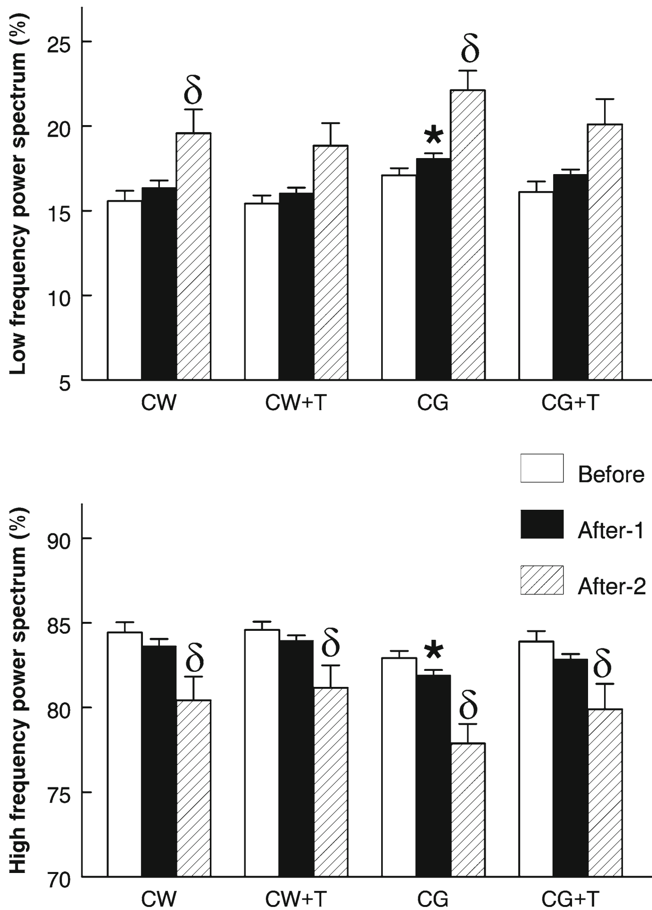

Fig. 2 Low (*upper*) and high (*lower*) frequency power spectral densities of arterial pressure pulse before (unconscious) and after (After-1, unconscious; After-2, conscious) cardiac ischemia/reperfusion ($^{\#,*}$δ $P<0.05$ compared to corresponding Before, CW, and After-1, respectively; *CW* control without high sugar intake ($n=7$); *CW+T* CW plus taurine supplementation ($n=8$), *CG* control with high sugar intake ($n=8$); *CG+T* CG plus taurine supplementation ($n=7$))

Sympathetic nerve activity-mediated regulation of arterial pressure, estimated by low frequency power spectral density of the arterial pressure pulse before cardiac IR, was not significantly different among the four groups (Fig. 2, upper). After cardiac IR, sympathetic activity tended to be higher in all unconscious compared to the corresponding before IR groups, the increase was statistically significant only in unconscious (but not conscious) CG compared to CW groups ($P<0.05$). Estimated parasympathetic nerve activity by high frequency power spectral density of arterial

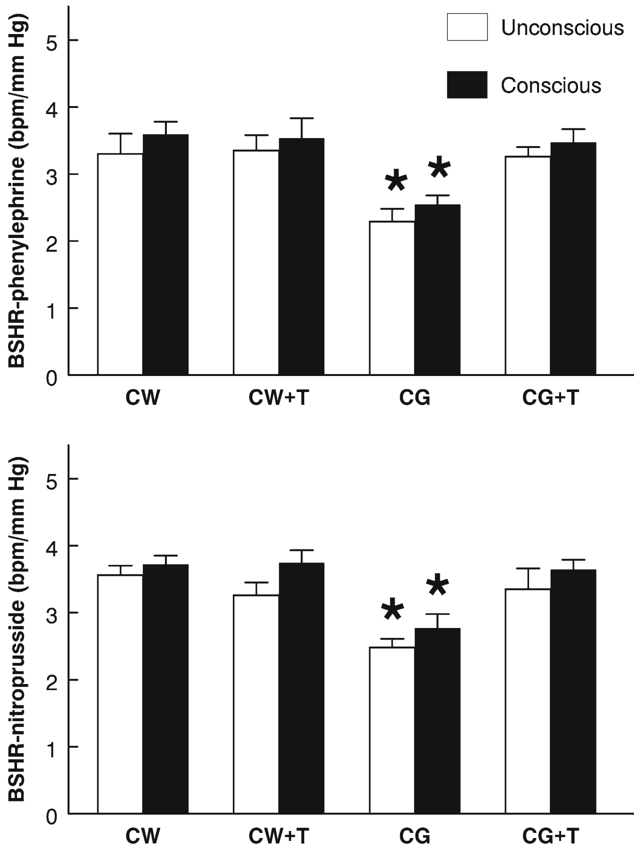

Fig. 3 Baroreflex sensitivity control of heart rate (BSHR) measured by phenylephrine infusion (*upper*) and sodium nitroprusside infusion (*lower*) after cardiac ischemia/reperfusion ($^{*}P<0.05$ compared to all other groups; *CW* control without high sugar intake ($n=7$), *CW+T* CW plus taurine supplementation ($n=8$); *CG* control with high sugar intake ($n=8$), *CG+T* CG plus taurine supplementation ($n=7$))

pressure pulse displayed the opposite response to the sympathetic nerve activity (Fig. 2, lower).

After cardiac IR, baroreflex sensitivity-mediated regulation of heart rate (Fig. 3) and renal nerve activity (Fig. 4) were significantly depressed in both conscious and unconscious CG compared to the other three groups (CW, CW+T, and CG+T). The baroreflex sensitivities measured in free moving and anesthetized rats were not significantly different among the paired groups. In addition, CW, CW+T, and CG+T groups displayed similar baroreflex sensitivities.

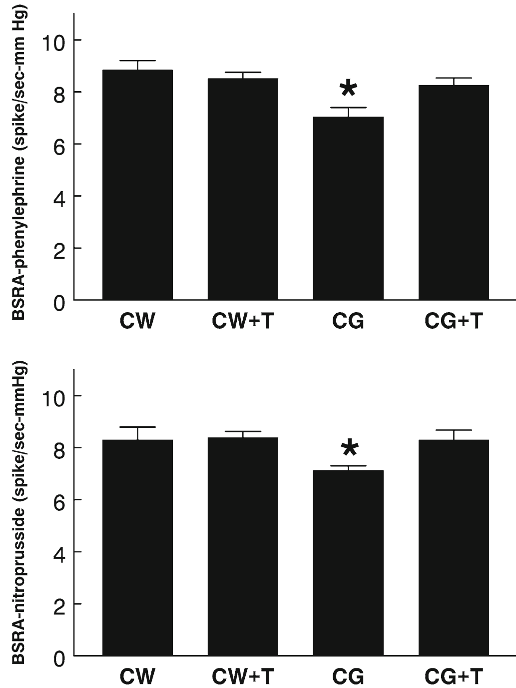

Fig. 4 Baroreflex sensitivity control of renal nerve activity (BSRA) measured by phenylephrine infusion (*upper*) and sodium nitroprusside infusion (*lower*) after cardiac ischemia/reperfusion (*$P<0.05$ compared to all other groups; *CW* control without high sugar intake ($n=7$), *CW+T*, CW plus taurine supplementation ($n=8$), *CG* control with high sugar intake ($n=8$), *CG+T* CG plus taurine supplementation ($n=7$))

4 Discussion

High sugar diets have been reported to increase the risk of cardiovascular disease in humans and animals. In the previous study, high sugar intake in adult male rats after weaning alters renal function before inducing hypertension and insulin resistance via renin-angiotensin system overactivation (Roysommuti et al. 2002). Although, the high sugar diet does not significantly alter autonomic and baroreflex function in normal male rats (Roysommuti et al. 2009), it significantly increases cardiac damage and induces baroreflex dysfunction after cardiac IR (Kulthinee et al. 2010).

The present study further indicates that in adult female rats, a high sugar diet depresses heart rate, baroreflex function, and parasympathetic nerve activity and increases sympathetic nerve activity after cardiac IR, without worsening cardiac injury. In addition, the present data indicate that short-term taurine supplementation starting a week before cardiac IR ameliorates cardiac injury independent of high sugar intake and abolishes the adverse effect of high sugar intake on bradycardia, autonomic function, and baroreflex control.

Baroreflex dysfunction is commonly observed after cardiac IR and predicts a poor prognosis (de La Fuente et al. 2013; Kulthinee et al. 2010; Rodrigues et al. 2011; Wang et al. 2000). Baroreflex dysfunction of the heart is usually due to an imbalance of sympathetic and parasympathetic control of the heart. In dogs, the

depression of cardiac baroreflex control shortly after cardiac IR can be prevented by preconditioning with a brief period of ischemia followed by reperfusion, and this beneficial effect is abolished by the muscarinic receptor blocker atropine (Babai et al. 2002). Further, atropine treatment without preconditioning does not affect the blunted baroreflex sensitivity after cardiac IR. These data suggest that sympathetic rather than parasympathetic nerve control dominates baroreflex control of the heart after cardiac IR.

Although our data in control female rats do not support the imbalance of sympathetic and parasympathetic nerve activity to the heart after cardiac IR compared to before IR episode, the increased sympathetic and decreased parasympathetic nerve activity observed in CG compared to CW groups suggests autonomic imbalance leads to the adverse effect of high sugar intake on baroreflex control after cardiac IR. However, the blunted baroreflex per se may also be due to intracardiac dysfunction after IR. The bradycardia observed in CG compared to CW groups despite heightened sympathetic and blunted parasympathetic nerve activity indicates that cardiac dysfunction after IR might be exacerbated by a high sugar diet leading to decreased cardiac sensitivity to autonomic stimulation.

The blunted baroreflex control of heart rate and renal nerve activity after cardiac IR seems to involve different mechanisms. Prostaglandins are released in the cardiac infarct area probably as a consequence of inflammation (Ustinova and Schultz 1994). Inhibition of prostaglandins by indomethacin treatment decreases the blunting of baroreflex control of renal nerve activity but not of heart rate control after cardiac IR in rabbits (Wang et al. 2000). Further, cardiac vagotomy also displays improved baroreflex sensitivity similar to that of indomethacin treatment. These data indicate that blunted baroreflex control of renal nerve activity after cardiac IR is mediated by prostaglandins acting within the heart, but also supports the evidence that the IR dysregulation of cardiac baroreflex control is mediated mainly by sympathetic nerve activity (Babai et al. 2002). Whether high sugar intake blunts baroreflex sensitivity after cardiac IR via these mechanisms requires further investigation. Nevertheless, decreased cardiac damage and increased baroreflex sensitivity resulting from taurine supplementation in CG+T but not CW+T groups suggests an inverse relationship between the degree of cardiac injury and baroreflex sensitivity in CG but not CW rats.

Increased sympathetic and decreased parasympathetic nerve activity after cardiac IR has been reported to be mediated by both intracardiac and extracardiac factors. Ischemia can locally stimulate cardiac sensory nerves to increase sympathetic nerve activity (Longhurst et al. 2001). Myocardial beta-adrenergic receptor overexpression (Bartels et al. 1998) and norepinephrine release (Fukumoto et al. 2012) are also observed after cardiac IR. Further, hypotension due to cardiac IR is known to mediate baroreceptor reflexes and stimulates the systemic renin-angiotensin system, thereby increasing sympathetic and decreasing parasympathetic nerve activity.

Although high sugar intake in the present study may not affect autonomic and renin-angiotensin system activity as previously reported (Thaeomor et al. 2013), it potentiates the sympathetic and blunts the parasympathetic nerve activity after cardiac IR. In other experiments, high sugar diets have been reported to stimulate

sympathetic nerve activity directly at the central nervous system level and indirectly via effects on the renin-angiotensin system, insulin resistance, and hyperinsulinemia (Freitas et al. 2007; Kopp 2005; Tran et al. 2009; Ward et al. 2011). Together with the findings that taurine supplementation completely abolishes the effect of high sugar intake on autonomic dysfunction and baroreflex sensitivity after cardiac IR and that taurine can inhibit these factors (Roysommuti and Wyss 2014), taurine is likely to antagonize the adverse effect of high sugar diet via these factors.

The present study induces cardiac IR by asphyxia to induce hypoxemia and hypotension; thus, the ischemia is global rather than local (Kulthinee et al. 2010). Among the four groups, the plasma NT-proBNP levels are released mainly by the cardiac ventricle (Bohm et al. 2011) and are less than 5 pg/l, suggesting that cardiac IR injury in the present study is not very severe. Trop-T and AST are synthesized mainly by extra cardiac tissues, particularly AST from the liver and Trop-T from skeletal muscle, while CK-MB is mainly synthesized by the myocardium (Bohm et al. 2011). Thus, the similar reduction in plasma CK-MB in taurine supplemented groups without any effect on plasma AST and Trop-T levels suggests taurine's effect is on the heart rather than on other tissues. In addition, the similar changes in plasma electrolytes, blood urea nitrogen, plasma creatinine, and hematocrit, irrespective of high sugar intake and taurine treatment make it unlikely that taurine acts via effects on electrolyte balance, hemodilution, and kidneys. However, the data do not exclude the potential effect of taurine with or without high sugar diets on the central nervous system and peripheral nerves (Roysommuti and Wyss 2014).

High sugar intake starting from weaning onwards does not affect sympathetic and parasympathetic nerve activity in male (Roysommuti et al. 2009) and female (Thaeomor et al. 2013) rats. However, this treatment depresses baroreflex sensitivity and increases cardiac injury after cardiac IR induction without any effect on autonomic activity in males (Kulthinee et al. 2010) and depresses baroreflex sensitivity, increases sympathetic and decreases parasympathetic activity after cardiac IR induction without any effect on cardiac injury in females (the present study). Lines of evidence indicate that females display lower severity of cardiac IR injury than males (Deschamps et al. 2010) and estrogen treatment prevents or improves cardiac IR injury (Kam et al. 2004). Acute inhibition of the estrogen receptor by tamoxifen depresses baroreflex sensitivity and parasympathetic activity and increases sympathetic activity to a similar extent in both CW and CG female rats (Thaeomor et al. 2013). Whether estrogen exerts an important action on baroreflex and autonomic function after cardiac IR has not been tested.

Taurine affects arterial pressure control via multiple mechanisms. High sugar intake induces hypertension that can be prevented or ameliorated by taurine supplementation (Feng et al. 2013; Harada et al. 2004; Nandhini and Anuradha 2004; Rahman et al. 2011). This antihypertensive action of taurine involves sympathetic inhibition, renin-angiotensin system suppression, decreased insulin resistance, antioxidation, and increased renal excretory function (Roysommuti and Wyss 2014). Taurine supplementation before and/or after cardiac IR episode prevents or improves cardiac injury potentially through many mechanisms, including antioxidation, cardiac renin-angiotensin system suppression, cardiac calcium balance, inhibition

of mitochondrial damage, and sympathetic inhibition (Schaffer et al. 2014b). In patients, taurine supplementation and diets high in taurine are recommended to counteract cardiovascular disease, particularly in patients at a high risk of cardiac ischemia/infarction and after cardiac surgery (Schaffer et al. 2014a).

5 Conclusion

High sugar intake affects arterial pressure control mechanisms and taurine supplementation can prevent or improve this adverse effect of high sugar intake. Although high sugar intake starting from weaning onwards may not affect autonomic and baroreflex functions in adult male and female rats, it can impair these functions in rats receiving cardiac IR. High sugar intake depresses baroreflex sensitivity and increases cardiac injury after cardiac IR induction without any effect on autonomic activity in males, while in females, it depresses baroreflex sensitivity, increases sympathetic and decreases parasympathetic activity without any effect on cardiac injury. Further, the present study indicates that short-term taurine supplementation prevents cardiac injury independent of high sugar intake and abolishes the adverse effect of high sugar intake on autonomic and baroreflex function, and on heart rate. These data support the hypothesis that taurine supplementation possesses a beneficial action on cardiac function and cardiovascular control after cardiac IR.

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Effects of Taurine on Blood Index of Hypothalamic Pituitary Adrenal (HPA) Axis of Stress-Induced Hypertensive Rat

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1 Introduction

Hypertension is a common and frequently occurring disease, and also one of the main causes of death. Environmental stresses stimulate the hypothalamus to secrete corticotropin releasing hormone (CRH), which acts through the hypothalamic-pituitary-adrenal (HPA) axis to induce the secretion of adrenocorticotropic hormone (ACTH), a hormone that promotes the secretion of glucocorticoid (GC) from the adrenal cortex. The elevation of blood pressure can be mediated by the central nervous system or the rostral ventrolateral medulla (Axelrod and Reisine 1984). Stress also initiates the classical kidney renin-angiotensin-aldosterone system (RAAS), with renin being secreted from activated glomerular artery juxtaglomerular cells. Renin cleaves angiotensinogen (AGT), generating angiotensin I (AngI), which is further converted to angiotensin II (Ang II) by angiotensin converting enzyme (ACE). Ang I can also be hydrolyzed to angiotensin 1–9 (Ang L 9), a reaction catalyzed by angiotensin converting enzyme 2 (ACE2) and involved in stress-induced hypertension (SIH) (Donoghue et al. 2000; Tipnis et al. 2000). Angiotensin II interacts with the angiotensin II receptor (ATR) to stimulate the generation of aldosterone, a hormone involved in sodium and water excretion by the adrenal cortex zona glomerulosa cells. Angiotensin II also stimulates arteriolar smooth muscle contraction. The above mechanisms can both elevate and maintain the high blood pressure (Sagesaka-Mitane et al. 2006). The RAAS system is one of the most important regulators of blood pressure, contributing to vasoconstriction, cation regulation and circulating blood volume (Struthers 1996). Indeed, the activation of RAAS system and the increase of plasma hormone may be the pathological basis for stress induced hypertension.

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However, stress also stimulates the hypothalamic neurosecretory cells to secrete corticotropin releasing hormone (CRH), which in turn stimulates the secretion of adrenocorticotropic hormone (ACTH) through the HPA axis and the secretion of glucocorticoid (GC). GC in turn stimulates the activity of phenylethanol amine-N-methyltransferase and inhibits the activity of catechol-O-methyltransferase (COMT), thereby affecting epinephrine and norepinephrine content. It also influences the expression of alpha adrenergic receptors and enhances the effect of catecholamines. GCs are also capable of decreasing the secretion of CRH by influencing the central nervous system (Yamamoto et al. 2006). They also can inhibit the synthesis of prostaglandin, 5-HT and histamine, which contribute to the degree of vasoconstriction. In addition, increased secretion of GC promotes renal tubular reabsorption and increases blood volume, which together elevate blood pressure. Thus, stress may represent an important mechanism by which blood pressure increases.

2 Methods

2.1 Materials

Angiotensin converting enzyme (ACE) kit, angiotensin converting enzyme 2 (ACE2) kit (EPI Kit), epinephrine, norepinephrine (NA) kit, angiotensin II (AngII) kit, Glucocorticoid (GC) kit, glucocorticoid hormone (CRH) ELISA kit, glucocorticoid hormone (CRH) kit, adrenocorticotropic hormone (ACTH) kit were purchased from Beijing Dingguo Biotechnology Co. Ltd.

2.2 Experimental Design

A total of 32 Male Wistar rats were randomly divided into 4 groups: normal control group, β-alanine stress group (β-alanine is an effective inhibitor of taurine transport that competes with taurine for the same receptor), untreated stress group, taurine stress group. Rats belonging to the untreated stress group and the normal control group were administered saline intragastrically at 8:00 a.m. while rats in the β-alanine stress group were administered β-alanine (200 mg/kg/day) intragastrically, with the aim of decreasing endogenous taurine. Rats in the taurine stress group were administered taurine (200 mg/kg/day) intragastrically. Stress was induced by foot shock and noise.

2.3 Establishment of Stress-Induced Hypertension Rats

As a source of stress, rats were exposed to discontinuous, irregular foot shocks (output voltage of 150 V every 5–10 s) and noise (100 dB of industrial noise) 2 h per day for 20 days. Systolic arterial pressure was measured between 8:30 and 10:00 a.m. the 1st, 6th, 11th, 16th and 21st day. After 21 days, all rats were killed, hypothalamus, pituitary and adrenal glands of rats were collected and stored at −80 °C.

2.4 Determination of Serum Samples

Serum was stored at low temperature were thawed at room temperature, then the content of serum epinephrine, norepinephrine, glucocorticoid, glucocorticoid hormone, nitric oxide synthase, T3, T4, AngII, ACE and ACE2 were assayed according to the kit instructions of Beijing Dingguo Biotechnology Co. Ltd.

2.5 Statistical Analysis

Statistical analysis was performed with SPSS17.0 statistical software, all the test data were presented as mean ± standard deviation. Multiple comparisons were carried by the LSD method. Figures were made using Excel.

3 Results

Through the combined stress of foot shock and noise for 21 days, blood pressure in the β-alanine stress group and the stress control group were significantly increased relative to that of the normal control group, suggesting that the establishment of stress-induced hypertension was successful. The results showed that blood pressure of rats in the β-alanine stress group increased from 118.93 ± 1.54 mmHg to 156.01 ± 2.51 mmHg, and the blood pressure of the untreated stress group was elevated from 120.08 ± 1.13 mmHg to 149.81 ± 3.08 mmHg, increases of 29.30 % and 23.59 % compared to those of the normal control group ($P < 0.05$) (Table 1). The blood pressure of the taurine stress group exhibited no significant difference with respect to the control group ($P > 0.05$). The blood pressure of the β-alanine stress group was significantly higher than that of the untreated stress group ($P < 0.05$) (Fig. 1).

Table 1 Tail artery blood pressure of rats in each group during the experiment

	Length of the stress time (mmHg)				
Group	1 day	6 day	11 day	16 day	21 day
Control group	120.65 ± 0.65[a]	122.17 ± 0.49[a]	124.60 ± 1.52[a]	125.65 ± 1.11[a]	128.25 ± 1.16[a]
β-Alanine stress group	118.94 ± 1.54[a]	129.01 ± 1.59[a]	141.23 ± 1.81[b]	151.39 ± 4.80[b]	156.01 ± 2.51[b]
Stress group	120.08 ± 1.13[a]	124.47 ± 3.18[a]	135.87 ± 2.47[c]	143.29 ± 2.02[c]	149.81 ± 3.08[c]
Taurine stress group	120.01 ± 0.96[a]	124.19 ± 0.94[a]	127.45 ± 2.49[a]	129.28 ± 1.52[a]	131.23 ± 1.09[a]

Note: Identical letters indicate insignificant difference between groups ($P > 0.05$). Different letters denote significant differences between groups ($P < 0.05$). The data shown represent means ± standard deviation

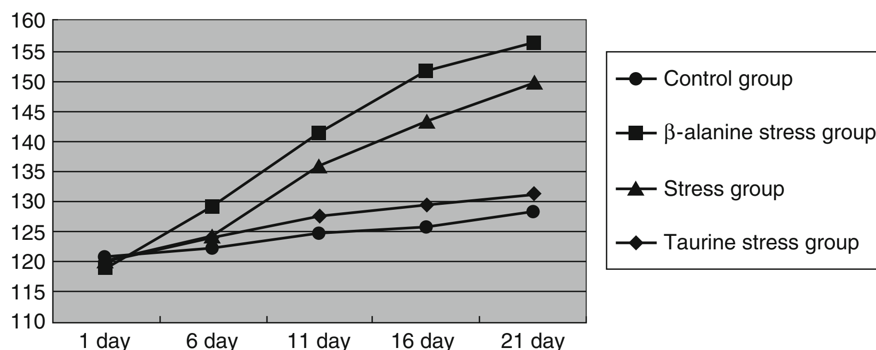

Fig. 1 Tail artery blood pressure of rats in each group during the experiment

Table 2 Effect of stress and taurine on serum ACE and ACE2 content

Group	ACE (U/L)	ACE2 (U/L)
Control group	28.45 ± 1.94[a]	18.50 ± 2.47[a]
β-Alanine stress group	37.91 ± 4.87[b]	19.22 ± 1.77[a]
Stress group	34.26 ± 3.40[b]	20.09 ± 1.45[a]
Taurine stress group	29.60 ± 1.85[a]	26.36 ± 3.72[b]

Identical letters indicate insignificant differences between groups ($P > 0.05$). Different letters indicate significant differences between groups ($P < 0.05$). The data shown represent means ± standard deviation

3.1 Determination of ACE and ACE2 Content in the Serum

The serum ACE results reveal obvious differences between the stress group and the normal control group ($P < 0.05$) (Table 2). Serum ACE content of the β-alanine stress group was significantly different from those of the normal control group and the taurine stress group ($P < 0.05$). The stress group and the taurine stress group also exhibited significant differences in serum ACE content ($P < 0.05$).

The results of serum ACE2 are shown in Table 2. Serum ACE2 content of the taurine stress group was significantly higher than those of the other groups ($P < 0.05$), while there were no obvious differences among the other three groups ($P > 0.05$).

3.2 Regulation of Serum NA and AngII Content

The effect of stress and taurine on serum norepinephrine (NA) is shown in Table 3. The stress group had significantly more serum NA than those of the normal control group and the taurine stress group ($P < 0.05$), while the β-alanine stress group exhibited significant differences with all of the other groups ($P < 0.05$).

Table 3 Effect of stress and taurine on serum NA and AngII content

Group	NA (ng/L)	AngII (ng/L)
Control group	41.66 ± 0.78[a]	39.06 ± 4.03[a]
β-Alanine stress group	56.04 ± 3.76[b]	62.72 ± 5.21[b]
Stress group	49.47 ± 2.02[c]	47.45 ± 2.72[c]
Taurine stress group	38.45 ± 0.46[a]	38.74 ± 2.53[a]

Same letter denotes insignificant difference between groups ($P > 0.05$). Different letters indicate significant differences between groups ($P < 0.05$). The data shown represent means ± standard deviation

There were no significant differences between serum angiotensin II (AngII) levels of the taurine stress group and the normal control group. Serum AngII content of the β-alanine stress group was significantly different from those of the normal control group and the taurine stress group ($P < 0.05$), but the AngII content of the stress group was not significantly different from those of the normal control, the β-alanine stress and the taurine stress groups ($P < 0.05$).

3.3 Regulation of Serum EPI, GC, CRH and ACTH by Stress

The taurine stress group showed no significant difference in serum EPI content compared with that of the normal control group, but did exhibit a significant difference relative to that of the stress group ($P > 0.05$). There was also a significant difference between serum EPI content of the stress group and that of the normal control group ($P < 0.05$). The β-alanine stress group showed no significant differences in serum EPI content compared with those of the normal control group and the taurine stress group ($P < 0.05$) (Table 4).

The results of serum GC of rats are shown in Table 4. The GC content of the taurine stress group was not significantly different from that of the normal group while the GC content of the stress group was significantly different from those of the other three groups ($P < 0.05$). The β-alanine stress group contained significantly higher levels of serum GC than those of the normal group and the taurine stress group ($P < 0.05$).

The results of ACTH content in rat serum are shown Table 4. The ACTH content of the taurine stress group was similar to that of the normal control group, it was significantly lower than that of the stress group ($P < 0.05$). The β-alanine stress group contained more serum ACTH than the other 3 groups ($P < 0.05$).

The serum of the taurine stress group contained comparable levels of CRH to that of the control group but was significantly less than those of the β-alanine stress and the untreated stress groups ($P < 0.05$). Although there were no obvious differences between the stress group and the normal control group ($P > 0.05$), taurine treatment abolished the effect of stress on CRH levels (Table 4).

Table 4 Effect of stress and taurine on serum EPI, GC, CRH and ACTH

Group	EPI (ng/L)	GC (ng/L)	ACTH (ng/L)	CRH (ng/L)
Control group	6.06 ± 0.20[a]	13.92 ± 1.69[a]	15.34 ± 0.88[a]	14.39 ± 0.36[a]
β-Alanine stress group	8.12 ± 0.98[b]	25.75 ± 1.11[b]	24.84 ± 0.74[b]	20.65 ± 2.45[b]
Stress group	7.42 ± 0.36[b]	22.93 ± 0.87[c]	20.34 ± 0.53[c]	19.42 ± 2.34[b]
Taurine stress group	5.98 ± 0.48[a]	14.33 ± 0.21[a]	15.89 ± 0.55[a]	14.91 ± 2.16[a]

Same letters denote insignificant differences between groups ($P > 0.05$). Different letters indicate significant difference between groups ($P < 0.05$). The data shown represent means ± standard deviation

Table 5 Effect of stress and taurine on serum T3 and T4 levels

Group	T3 (ng/L)	T4 (μg/L)
Control group	21.73 ± 3.16[a]	1.78 ± 0.09[a]
β-Alanine stress group	12.89 ± 2.61[b]	1.64 ± 0.38[a]
Stress group	13.56 ± 1.28[b]	2.66 ± 2.62[a]
Taurine stress group	19.18 ± 1.34[a]	2.01 ± 1.13[a]

3.4 Determination of T3 and T4 Content in the Serum

T3 content of the β-alanine stress group was significantly lower than those of the normal control group and of the taurine stress group ($P < 0.05$), but there was no significant difference between the untreated and the β-alanine treated stress groups ($P > 0.05$). There were no significant differences between the taurine stress group and the normal control group ($P > 0.05$). The T4 content of all four groups was statistically indistinguishable (Table 5) ($P > 0.05$).

4 Discussion

4.1 Effect of Taurine on the RAAS in Stressed Rats

Epinephrine and norepinephrine can stimulate the release of renin, whereas angiotensin, vasopressin, atrial natriuretic peptide, endothelin and NO inhibit its release. The content of norepinephrine in the β-alanine stressed rats was higher than those of the other groups, indicating that the β-alanine stress group, which is also a taurine deficiency group, stimulates RAAS (Gallagher et al. 2003). Angiotensin II is known to enhance the release of norepinephrine from sympathetic nerve endings, resulting in increased peripheral vascular resistance and blood pressure. In summary, the antihypertensive actions of taurine are related to an intervention in RAAS, likely through elevations in ACE2, inhibition of AngI directly or reducing the synthesis of AngI, thereby inducing vasoconstriction and reducing total peripheral vascular resistance and blood pressure.

This study found that exposure to intermittent noise and foot shock led to an increase in blood pressure in rats. Treatment with β-alanine combined with the stress of foot shock and noise significantly increases serum norepinephrine and angiotensin II levels, indicating that RAAS has been activated. By contrast, taurine treatment attenuated the activation of the RAAS in rats exposed to foot shock and noise (Iyer et al. 2000). It is noted that ACE2 activity is highest in the serum of the taurine stress group while ACE declines in the taurine stress group, suggesting that taurine-mediated alterations in blood pressure in the taurine stress group is mediated by a reduction in AC levels and an increase in ACE2 levels.

4.2 Effects of Taurine on Hypothalamic Pituitary Adrenal (HPA) Axis and the Hypothalamic Pituitary Thyroid Axis in Stress Rats

The influence of multiple factors, including central nervous stimulation, induce the release of corticotropin releasing hormone from the hypothalmus, which could combine with vasopressin to promote the release of adrenocorticotropic hormone (ACTH), which in turn acts on the adrenal cortex. Glucocorticoids (mainly cortisol) are synthesized by the adrenal cortex under the regulation of ACTH. The stimulation of the sympathetic nervous system and the elevation in cortisol together stimulate the synthesis and secretion of adrenaline and noradrenaline from the adrenal medulla, which interact synergistically with RAAS. Also influencing the response to stress are adrenaline, which is a short-term stress hormone, and thyroxine (T3 and T4), which is a long-term stress hormone (Ferrario 2003).

Chronic stress can significantly inhibit the function of the hypothalamus-pituitary-thyroid axis, thereby reducing the serum levels of thyroid hormone. Under acute stress, which leads to the excitement of the sympathetic nervous system, the secretion of TRH from the hypothalamus and enhanced TSH synthesis by the pituitary, thereby increasing the release of thyroid hormone from the thyroid gland into the blood. Thyroid hormone is known to accelerate the rate of energy metabolism in order to meet the energy needs of tissues (DiezFreire et al. 2006). Cold and heat stress primarily act through the hypothalamus–pituitary–thyroid axis to elevate thyroid hormone. However, chronic stress inhibits the function of the hypothalamus–pituitary–thyroid axis. Indeed, T3 and TSH levels of chronically stressed elderly patients are significantly decreased (Struthers 1996). The development of this phenomenon may be associated with reduced secretion of thyroid releasing hormone (TRH) and cytokine-mediated inhibition of TSH secretion.

The results of this study show that T3 content in the β-alanine stress group was significantly lower than that of the normal control group. The reason may be that under chronic stress, taurine deficient rats lack the ability to resist stress, leaving the immune system in a compromised state (Danilczyk and Penninger 2006). The reduction in the synthesis of TRH in the hypothalamus influences the synthesis of

TSH in the pituitary, thereby reducing the synthesis of T3 in the thyroid. However, there were no differences in T4 content among the groups, perhaps because the function or the activity of the enzyme converting T4 to T3 is either reduced or inhibited.

Chronic stressors excite the sympathetic adrenal medulla system, so that the secretion of catecholamines, such as epinephrine (EPI) and norepinephrine (NA) increase, leading to the enhancement of the body's defense capability, as well as the body's energy consumption and blood pressure. However, the end result of chronic stress is system dysfunction. Thus, two measures are taken to reduce chronic multiple stress, one involves management and treatment to reduce the source of exogenous stress stimuli and the other is to improve the anti-stress ability of the body. Combining those two aspects, the secretion of EPI, NA, CHR, ATCH and GC will achieve a stable and reasonable level.

Cardiomyocytes of hypertrophic rats exhibit altered taurine transmembrane transport function, perhaps related to the downregulation of the TAUT gene (Zielinska et al. 1999). Therefore, only exogenous supplementation can stabilize the amount of taurine in vivo (Shi et al. 2002). Taurine protects the cell membrane against oxidant, free radical damage and viral injury (Huxtable 1992). Taurine appears to limit oxygen free radical production and resist lipid peroxidation damage. Thus, exogenous supplementation of taurine effectively corrects endogenous transport barriers and corrects changes in body function.

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Taurine in 24-h Urine Samples Is Inversely Related to Cardiovascular Risks of Middle Aged Subjects in 50 Populations of the World

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Abbreviations

TAU	Taurine
CHD	Coronary heart diseases
Cre	Creatinine

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BMI	Body mass index
BP	Blood pressure
TC	Total cholesterol
CARDIAC	Cardiovascular Diseases and Alimentary Comparison
SHR	Spontaneously hypertensive rats
Na	Sodium
K	Potassium
Mg	Magnesium
ORs	Odds ratios
CYP7A	Cytochrome P450 7A1
LDL	Low-density lipoprotein

1 Introduction

Taurine (Tau), which is rich in various seafood such as fish, shells, squid, and shrimp, is a ubiquitous sulfur-containing amino acid involved in many important biological functions (Huxtable 1992). The preventive effect of dietary Tau against hypertension and stroke was first proven experimentally in rat models of genetic hypertension, spontaneously hypertensive rats (SHR) and stroke-prone SHR (Nara et al. 1978; Okamoto and Aoki 1963; Okamoto et al. 1974). Further, experimental and clinical studies examining the effect of Tau on hypertension (Fujita and Sato 1986; Fujita et al. 1987; Yamori et al. 2009), dyslipidemia (Murakami et al. 1996; Yamori et al. 2010a; Yokogoshi et al. 1999), atherosclerosis (Murakami 2014; Murakami et al. 1996, 2002a, 2010) and obesity (Fujihira et al. 1970; Tsuboyama-Kasaoka et al. 2006; Zhang et al. 2004) supported a possible role for Tau in reducting cardiometabolic diseases. A world-wide epidemiological survey to investigate the association of nutritional biomarkers in 24-h urine samples, including sodium (Na), potassium (K), Magnesium (Mg), and Tau with cardiovascular risk factors was carried out by WHO-coordinated cardiovascular Diseases and Alimentary Comparison (CARDIAC) Study in over 60 populations of the world (WHO-CARDIAC Study group 1986, 1990; Yamori et al. 1990, 1996, 2001, 2006, 2010b).

This study showed an inverse association between average 24-h urinary Tau excretion and the mortality due to coronary heart disease (CHD) (Yamori et al. 1996, 2001, 2006) and also reported that the group of individuals excreting more than the world average of 24-h urinary taurine (Tau/creatinine (Cre) ratio) had significantly lower cardiovascular disease risks (Yamori et al. 2010b), and therefore Tau/Cre ratios are inversely associated with CHD and stroke (Yamori et al. 2010b). This study confirmed that there was a close relationship between the population averages of cardiovascular disease risks and the dietary habits, established by analyzing various dietary biomarkers in 24-h urine samples (Yamori et al. 1984). However, the population averages are influenced greatly by genetic background of the individuals and environmental factors in the regions examined. Therefore, in the

present study, we investigated the association with and without the adjustment of confounding variables between 24-h urinary Tau/Cre ratio and cardiovascular disease risk factors in individuals, including obesity, hypertension and hypercholesterolemia, among the CARDIAC study populations disregarding genetic background, living conditions and gender.

2 Methods

2.1 Study Population

The WHO-coordinated CARDIAC Study was initiated in 1985 as a multi-center cross-sectional study with a standard research protocol, with a total of 12,335 men and women participating in the study. Details of the study design and methods of the CARDIAC Study have been reported elsewhere (WHO-CARDIAC Study group 1986, 1990; Yamori et al. 1990, 2006). Briefly, in each center, 100 men and 100 women aged 48–56 years were selected randomly from the general population. In the present study, 50 population samples of 22 countries are included. These 22 countries include various ethnic groups and diverse populations: Australia (97 participants), Brazil (244), Belgium (165), Bulgaria (209), Canada (160), China (686), Ecuador (254), France (158), Georgia (65), Greece (35), Israel (50), Italy (82), Japan (920), New Zealand (140), Portugal (115), Russia (31), Spain (274), Sweden (28), Tanzania (51), Nigeria (40), UK (224) and USA (183) (Fig. 1). The study was approved by the CARDIAC Study's institutional review board committee.

2.2 Data Collection

After excluding the participants who had missing data or who failed to complete the 24-h urine collection, the remaining 4,211 participants (2,120 men and 2,091 women) were included in data analyses. All participants were invited to a local hospital or health center for a physical examination, and a 15-ml overnight fasting blood sample was obtained. 24-h urine samples were collected using a standard aliquot cup that allowed participants to collect an exact portion of voided urine repeatedly (WHO-CARDIAC Study group 1986, 1990). BP was measured using a standard automated sphygmomanometer (Khi machine, VINE Co., Ltd., Tokyo) and these measurements were repeated three times (Fukuda and Yamori 1987; WHO-CARDIAC Study group 1986, 1990). A structured questionnaire was used for face-to-face interviews during the field survey, and included items on demographic data, lifestyle factors and medical history (WHO-CARDIAC Study group 1986, 1990). The urine and blood samples were frozen at −20 °C and analyzed

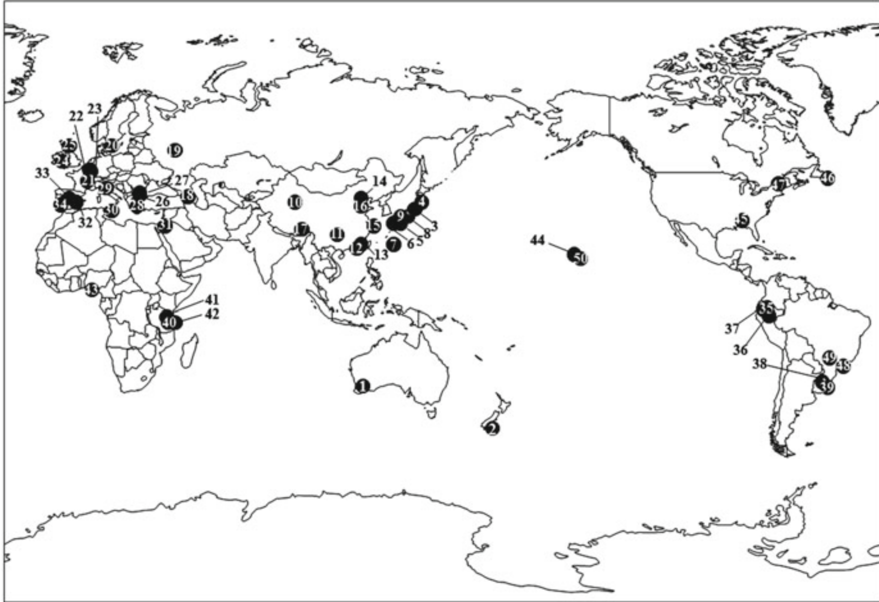

Fig. 1 Geographical distribution of population samples of the Cardiovascular Disease and Alimentary Comparison (CARDIAC) Study (1985–1994). The following are the 50 study sites of 22 countries and their principal investigators. (*1*) Perth: L.J. Beilin, M.S.T. Hobbs, K. Jamrozik. (*2*) Dunedin: F.O. Simpson. (*3*) Toyama: S. Kagamimori. (*4*) Hirosaki: T. Kanazawa. (*5*) Beppu: S. Kodama. (*6*) Kurume: H. Toshima. (*7*) Okinawa: G. Mimura, K. Taira. (*8*) Hiroshima: M. Yamakido. (*9*) Ohda: Y. Yamori. (*10*) Urumqi: B.X. He. (*11*) Guiyang: M.X. Zhang, X.L. Wu. (*12*) Guangzhou: Z.D. Huang. (*13*) Meshen: I. Lee. (*14*) Beijing: L.S. Liu. (*15*) Shanghai: G.S. Zhao. (*16*) Shijiazhuang: H.X. Zhang. (*17*) Lhasa: S.F. Sun. (*18*) Georgia: S.M. Dalakishivili. (*19*) Moscow: R.G. Oganov. (*20*) Gothenburg: L. Wilhelmsen. (*21*) Orleans: A. Marie. (*22*) Leuven: A. Amery. (*23*) Ghent: G. De Backer. (*24*) Belfast: A.E. Evans. (*25*) Stornoway: C.A. Birt. (*26*) Sofia (urban): N. Nicolov, I. Tomov. (*27*) Sofia (rural): N. Nicolov, I. Tomov. (*28*) Athens: A. Ioanidis. (*29*) Milan: G. Cerasola. (*30*) Palermo: G.C. Cesana. (*31*) Tel Aviv: T. Rosenthal. (*32*) Navas: A. Fernandez-Cruz. (*33*) Madrid: A. Fernandez-Cruz. (*34*) Lisbon: M.O. Carrageta. (*35*) Quito: P.D. Dillon. (*36*) Vilcabamba: V. Del Pozo. (*37*) Manta: V. Del Pozo. (*38*) Uruguaiana: Y. Moriguchi, E. Moriguchi. (*39*) Bagé: Y. Moriguchi, E. Moriguchi. (*40*) Handeni: J. Mtabaji, M. Njelekela. (*41*) Shinya: J. Mtabaji, M. Njelekela. (*42*) Dar es Salaam: J. Mtabaji, M. Njelekela. (*43*) Ibadan: O.O. Akinkungbe. (*44*) Honolulu: G. Mimura. (*45*) Jackson: H.G. Langford. (*46*) Newfoundland: G. Fodor, A. Chockalingam. (*47*) Montreal: P. Hamet. (*48*) Sao Paulo: Y. Moriguchi, E. Moriguchi. (*49*) Campo Grande: Y. Moriguchi, E. Moriguchi. (*50*) Hilo: M. Kanahele

centrally in the laboratory of WHO-collaborating Center for Research on Primary Prevention of Cardiovascular Disease, Izumo, Japan (in 1993, this center was transferred to the Graduate School of Human and Environmental Studies, Kyoto University, Japan). Standardized laboratory methods were used (WHO-CARDIAC Study group 1986, 1990). Quality controls were carefully maintained by internal and external quality surveillance procedures. Measurements included in the present report are BMI, BP, serum total cholesterol (TC), urinary Na, K, Mg, Ca, Cre and Tau excretion levels.

2.3 Statistical Analysis

Subjects with obesity were defined as those with BMI ≥ 30 kg/m^2. Patients with hypertension were defined as those with systolic BP ≥ 140 mmHg or diastolic BP ≥ 90 mmHg or those who were receiving anti-hypertensive drug therapy. Hypercholesterolemic subjects were defined as those with serum TC ≥ 220 mg/dl. The markers in 24-h urine are expressed as the ratio of each parameter relative to Cre. We categorized 24-h urinary Tau/Cre ratio in quintiles. The distributions of 24-h urinary Na/Cre, K/Cre, Ca/Cre and Mg/Cre ratio were highly skewed, and thus log transformations were performed to achieve a normal distribution. In all analyses, the log-transformed values were then used. For easy interpretation, nontransformed values are reported in the tables.

Differences between baseline characteristics of participants within each quintile were analyzed using the Cochrane-Armitage test for trends for proportions and the Jonckheere-Terpstra trend test for continuous measures. General linear models were used to estimate adjusted means of BMI, systolic BP, diastolic BP and TC across quintiles of 24-h urinary Tau/Cre ratio after adjustment for potential confounding variables. Models were initially adjusted for potential confounders by traditional cardiac risk factors (age, sex and use of anti-hypertensive medication). Final multivariable models were additionally adjusted for natural logarithm-transformed 24-h urinary Na/Cre, K/Cre, Ca/Cre, Mg/Cre ratios and survey years (1985–1989 or 1990–1994, which represent participants who had an average age of 52 years old and were born in the years 1933–1937 and 1938–1942, in order to adjust potential cohort effects on the study outcomes).

To evaluate the association between the Tau/Cre ratio and cardiovascular disease risk factors, we estimated adjusted odds ratios (ORs) for obesity, hypertension and hypercholesterolemia in relation to quintiles of the Tau/Cre ratio using logistic regression models, including variables for age, sex, anti-hypertensive medication use, natural logarithm-transformed Na/Cre, K/Cre, Ca/Cre, Mg/Cre ratios and survey year (1985–1989 or 1990–1994). Adjustments were made in two stages the same as the analyses of multiple linear regression models described above. Anti-hypertensive medication use was not included in the adjusted variables for the estimation of ORs for hypertension.

All statistical analyses except for the Cochrane-Armitage test for trends were conducted using SPSS 15.0J for Windows (IBM Japan, Tokyo, Japan). The Cochrane-Armitage test for trends was performed using EXCEL 2003 (Microsoft, Tokyo, Japan). A two-sided P value ≤0.05 was considered statistically significant.

3 Results

Mean values and proportions of each characteristic by quintiles of 24-h urinary Tau/Cre ratio are shown in Table 1. There was a 14.8-fold difference in the Tau/Cre ratio between the highest and lowest quintiles of the study population (medians:

Table 1 Baseline characteristics by quintiles of 24-h urinary taurine/creatinine ratio in the CARDIAC Study, 1985–1994

	Quintile of taurine/creatinine ratio (μmol/mmol)					
	1 (lowest) (n=842, Median, 16.0; Range, 0.8–<28.8)	2 (n=842, Median, 41.9; Range, 28.8–<55.7)	3 (n=843, Median, 72.7; Range, 55.7–<93.7)	4 (n=842, Median, 121.7; Range, 93.7–<161.9)	5 (high) (n=842, Median, 236.5; Range, ≥162.0)	*P* for trend[a]
Male (%)	42.8	56.2	57.4	49.8	45.6	<0.001
Age (years)	51.9(1.7)	51.8 (1.7)	51.8(1.6)	51.9 (1.7)	52.2 (1.7)	<0.01
Body mass index (kg/m^2)	26.1 (4.3)	25.5 (4.0)	24.5 (3.9)	24.3 (4.1)	23.9 (3.8)	<0.001
Systolic blood pressure (mmHg)	125.5 (19.3)	125.7 (19.8)	123.8 (19.0)	123.8 (19.5)	123.6 (20.2)	<0.01
Diastolic blood pressure (mmHg)	75.1 (12.1)	75.9 (12.5)	74.9 (12.5)	73.6 (12.2)	74.1 (13.1)	<0.001
Serum total cholesterol (mg/dl)	194.5 (48.6)	197.1 (45.8)	189.2 (49.5)	183.3 (40.8)	181.5 (41.6)	<0.001
Antihypertensive treatment (%)	5.6	4.4	4.4	4.5	3.3	<0.001
Obesity[b] (%)	12.8	11.5	9.9	10.2	6.5	<0.001
Hypertension[c] (%)	27.7	26.2	23.7	22.8	24.2	<0.001
Hypercholesterolemia[d] (%)	28.4	28.9	23.1	17.7	15.2	<0.001
Nutrient marker in 24-h urine						
Magnesium/creatinine (mg/mg)	77.9 (39.4)	74.5 (39.4)	78.1 (44.0)	80.4 (37.5)	81.3 (41.4)	<0.001
Calcium creatinine (mg/mg)	135.2 (76.5)	136.1 (87.6)	145.8 (82.7)	162.3 (92.6)	180.5 (94.0)	<0.001
Sodium/creatinine (mmol/mmol)	16.1 (7.8)	15.7(7.3)	17.1 (8.0)	18.3 (8.2)	21.0 (8.9)	<0.001
Potassium/creatinine (mmol/mmol)	5.4 (2.1)	5.0 (2.0)	4.8 (2.0)	4.9 (2.2)	5.4 (2.4)	<0.05
Taurine/creatinine (μmol/mmol)	15.7(7.7)	42.1 (7.7)	73.6 (11.2)	123.3 (19.7)	299.4 (188.7)	<0.001

Values are expressed as mean (standard deviation) or percentage
[a]P values were determined by the Cochrane-Armitage test for trend for proportions and the Jonckheere-Terpstra trend test for continuous measures
[b]Obesity was defined as body mass index ≥30 kg/m^2
[c]Hypertension was defined as an anti-hypertensive treatment or systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg
[d]Hypercholesterolemia was defined as serum total cholesterol ≥220 mg/dl

236.5 μmol/mmol/day in the highest quintile, 16.0 μmol/mmol/day in the lowest). There was a weak association between the Tau/Cre ratio and mean age, while higher Tau/Cre ratio was significantly associated with a lower percentage of obesity, hypertension and hypercholesterolemia and with higher Na/Cre, K/Cre, Ca/Cre and Mg/Cre ratios. Participants with the lower Tau/Cre ratio were more likely to be female, obese, hypertensive or hypercholesterolemic than participants with a higher Tau/Cre ratio.

Table 2 shows the adjusted mean values of cardiovascular disease risk factors by the quintiles of the 24-h urinary Tau/Cre ratio. The Tau/Cre ratio was inversely associated with BMI, diastolic BP and TC. Adjustment of the analysis of age, sex and anti-hypertensive drug use in Model 1 did not markedly attenuate the associations of Tau/Cre ratio with BMI and TC, and enhanced the association of the ratio with systolic BP and diastolic BP. Upon further adjustment for Na/Cre, K/Cre, Ca/Cre, Mg/Cre ratios and survey year relative to BMI, SBP, DBP and TC in Model 2, the inverse trends remained significant for all the parameters (P for linear trend <0.001 for all comparisons across quintiles).

ORs for obesity, hypertension and hypercholesterolemia are presented in Table 3 for each quintile of the 24-h urinary Tau/Cre ratio. Tau/Cre ratio was significantly associated with obesity and hypercholesterolemia in the crude analysis. The prevalence of obesity and hypercholesterolemia increased in a dose-dependent manner from the highest quintile to the lowest quintile of the Tau/Cre ratio. Participants with the lowest Tau/Cre ratio were 2.91 and 2.21 times more likely to be obese and hypercholesterolemic, respectively, than those within the highest quintile of the Tau/Cre ratio. After adjusting for age, sex and antihypertensive drug use in Model 1, inverse association between Tau/Cre and the prevalence of hypertension became significant. Further adjustment for the confounding variables of other urinary biomarkers and survey years in Model 2 did not markedly change these inverse associations. In the analyses of the prevalence of hypertension, participants with the lowest Tau/Cre ratio were 1.22 and 1.29 times more likely to have hypertension than those within the highest quintile of the Tau/Cre ratio in the analysis of Model 1 and 2. However, the risk of hypertension among the subjects within the second to fourth quintiles was not significantly high compared with that in the subjects in the highest quintile.

4 Discussion

In this multi-center cross-sectional study, the quintile analysis of the 24-h urinary Tau/Cre ratios indicated that the ratios were inversely associated with obesity, hypertension, hypercholesterolemia and nutrient markers in 24-h urine, such as Mg/Cre, Ca/Cre, Na/Cre, K/Cre and Tau/Cre. In linear regression analyses, the Tau/Cre ratios were inversely associated with BMI, diastolic BP and TC. After adjustment for age, sex and antihypertensive drug use, the inverse association of Tau/Cre with systolic BP became significant. The further adjustment for all available 24-h urinary biomarkers did not markedly change these associations.

Table 2 Adjusted means and 95 % confidence intervals of body mass index, blood pressure, serum total cholesterol according to the levels of 24-h urinary taurine/creatinine ratio in the CARDIAC Study, 1985–1994

	Quintile of taurine/creatinine ratio (μmol/mmol)					
	1 (lowest) (n = 842, Median, 16.0; Range, 0.8–<28.8)	2 (n = 842, Median, 41.9; Range, 28.8–<55.7)	3 (n = 843, Median, 72.7; Range, 55.7–<93.7)	4 (n = 842, Median, 121.7; Range, 93.7–<161.9)	5 (high) (n = 842, Median, 236.5; Range, ≥162.0)	P for linear trend[a]
Body mass index (kg/m^2)						
Crude	26.1 (25.4, 26.7)	25.5 (24.9, 26.2)	24.5 (23.9, 25.2)	24.3 (23.6, 24.9)	23.9 (23.6, 24.2)	<0.001
Model 1[b]	26.0 (25.4, 26.7)	25.5 (24.9, 26.2)	24.6 (23.9, 25.2)	24.3 (23.6, 24.9)	23.9 (23.7, 24.2)	<0.001
Model 2[c]	26.0 (25.3, 26.6)	25.4(24.8, 26.1)	24.6 (24.0, 25.3)	24.3 (23.7, 25.0)	24.0 (23.7, 24.2)	<0.001
Systolic blood pressure (mmHg)						
Crude	125.5 (122.3, 128.7)	125.7 (122.5, 128.9)	123.8 (120.6, 127.0)	123.8 (120.6, 127.0)	123.6 (122.3, 124.9)	NS
Model 1[b]	125.5 (122.4, 128.6)	125.7 (122.6, 128.9)	123.8 (120.6, 126.9)	123.8 (120.7, 127.0)	123.6 (122.3, 124.9)	<0.001
Model 2[c]	126.1 (122.8, 129.4)	126.0 (122.7, 129.2)	123.8 (120.6, 127.0)	123.6 (120.5, 126.8)	123.0 (121.6, 124.3)	<0.001
Diastolic blood pressure (mmHg)						
Crude	75.1 (73.1, 77.2)	75.9 (73.9, 78.0)	74.9 (72.8, 76.9)	73.6 (71.6, 75.7)	74.1 (73.3, 75.0)	<0.01
Model 1[b]	75.3 (73.3, 77.3)	75.8 (73.8, 77.8)	74.7 (72.7, 76.7)	73.6 (71.6, 75.6)	74.3 (73.5, 75.1)	<0.001
Model 2[c]	75.4 (73.3, 77.5)	75.8 (73.7, 77.8)	74.7 (72.7, 76.8)	73.7 (71.7, 75.7)	74.1 (73.3, 75.0)	<0.001
Serum total cholesterol (mg/dl)						
Crude	194.5 (187.0, 201.9)	197.1 (189.7, 204.5)	189.2 (181.8, 196.6)	183.3 (175.9, 190.7)	181.5 (178.4, 184.6)	<0.001
Model 1[b]	194.0 (186.6, 201.4)	197.5 (190.1, 204.9)	189.7 (182.2, 197.1)	183.3 (175.9, 190.7)	181.1 (178.1, 184.2)	<0.001
Model 2[c]	192.6 (185.2, 199.9)	196.1 (188.8, 203.4)	190.8 (183.6, 198.1)	184.7 (177.5, 191.8)	181.4 (178.4, 184.4)	<0.001

[a]From multiple linear regression models for the relation between 24-h urinary magnesium/creatinine ratio and the risk factors of cardiovascular diseases
[b]Model 1 was adjusted for age, sex and use of anti-hypertensive drugs
[c]Model 2 additionally adjusted for 24-h urinary sodium/creatinine, potassium/creatinine, calcium/creatinine, magnesium/creatinine and survey year

Table 3 ORs (and 95 % CIs) for the prevalence of cardiovascular disease risk factors according to the levels of 24-h urinary Tau/Cre ratio in the CARDIAC Study, 1985–1994

	Quintile of taurine/creatinine ratio (Tau/Cre)									P for linear trend[a]
	1 (lowest)		2		3		4		5 (highest)	
Obesity[b]										
Crude	2.91	(2.09, 4.04)[c]	1.83	(1.29, 2.60)	1.14	(0.78, 1.66)	1.53	(1.07, 2.19)	1.00	<0.001
Model 1[d]	2.84	(2.04, 3.96)	1.87	(1.32, 2.66)	1.16	(0.80, 1.71)	1.52	(1.07, 2.20)	1.00	<0.001
Model 2[e]	2.91	(2.05, 4.12)	1.83	(1.27, 2.63)	1.15	(0.78, 1.70)	1.52	(1.06, 2.19)	1.00	<0.001
Hypertension[b]										
Crude	1.19	(0.96, 1.49)	1.11	(0.89, 1.38)	0.97	(0.78, 1.21)	0.92	(0.74, 1.16)	1.00	NS
Model 1[d]	1.22	(0.98, 1.51)	1.11	(0.89, 1.39)	0.97	(0.78, 1.22)	0.93	(0.74, 1.17)	1.00	<0.05
Model 2[e]	1.29	(1.02, 1.62)	1.15	(0.91, 1.44)	0.99	(0.79, 1.25)	0.94	(0.75, 1.18)	1.00	<0.01
Hypercholesterolemia[b]										
Crude	2.21	(1.74, 2.81)	2.26	(1.78, 2.87)	1.68	(1.31, 2.15)	1.20	(0.93, 1.55)	1.00	<0.001
Model 1[d]	2.20	(1.73, 2.80)	2.36	(1.85, 3.01)	1.76	(1.37, 2.25)	1.22	(0.94. 1.58)	1.00	<0.001
Model 2[e]	1.94	(1.49, 2.53)	2.12	(1.64, 2.75)	1.74	(1.34, 2.26)	1.22	(0.93, 1.60)	1.00	<0.001

[a]Logistic regression analysis evaluating the linear relations between 24-h urinary taurine/creatinine ratio and cardiovascular diseases risk factors
[b]Obesity, hypertension and hypercholesterolemia were defined as body mass index ≥30 kg/m^2, any anti-hypertensive medication use or systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg, and serum total cholesterol ≥220 mg/dl, respectively
[c]Odds ratio; 95 % CI in parentheses (all such values)
[d]Model 1 was adjusted for age and sex as to hypertension and additionally adjusted for anti-hypertensive medication use as to obesity and hypercholesterolemia
[e]Model 2 additionally adjusted for log-transformed 24-h urinary sodium/creatinine, potassium/creatinine, calcium/creatinine, magnesium/creatinine and survey year

Previous studies using data from the WHO-CARDIAC study demonstrated that the population averages of Tau in the 24-h urine samples were inversely related with CHD mortality rates (Yamori et al. 1996, 2001, 2006). Further analysis of individual 24-h urine samples from 41 populations of the WHO-CARDIAC Study revealed that individuals with 24-h Tau/Cre and Mg/Cre ratios more than the average of all the CARDIAC samples had significantly lower BMI, systolic and diastolic BP and TC than those with the ratios below the average, despite differences in ethnicity and genetic background (Yamori et al. 2010a, b). The present study confirmed and extends our previous findings by testing the associations of quintile scales of the Tau/Cre ratio with three key CVD risk factors and with or without the adjustment for more confounding variables, including Mg/Cre in multivariate analyses.

The role of Tau in BP regulation was first noted by its antihypertensive effect in SHR and stroke-prone SHR (Nara et al. 1978; Yamori 1984a, b, 1989), with the antihypertensive mechanism being due to sympathetic modulation in rat models (Li et al. 1996) and humans (Mizushima et al. 1996). Clinically, Tau administration decreased BP in borderline hypertensive young patients (Fujita et al. 1987) and in Tibetans, which exhibited nearly the lowest 24-h urinary Tau excretion among CARDIAC Study populations in the world because of their religious discipline not to eat fish (Yamori et al. 2009). CARDIAC Study demonstrated populations with greater 24-h Tau excretion in man had significantly lower BP and slower heart rates (HR) than those with lower Tau excretion (Yamori et al. 2009). Further analysis of CARDIAC data world-wide revealed salt-induced BP rise, that is the salt sensitivity was observed in individuals excreting higher Na accompanied by higher HR, among whom higher 24-h urinary Tau excretion was associated with lower BP. These data indicate possible neural involvement in salt-sensitive hypertension and suggest enough Tau intake attenuates salt-induced BP rise.

Quintile analyses of this study indicated an association between obesity in the lowest Tau excretion group, which was 2.9 times more than in subjects with the highest taurine excretion rates, with or without adjustment for confounding variables. Tau supplementation decreased body weight and abdominal fat experimentally in obese KK mice (Fujihira et al. 1970) and body weight clinically in over weight subjects (Zhang et al. 2004). Tau synthesis was reported to be decreased in white adipose tissue of obese mice due to a reduction in cysteine deoxygenate expression, a rate limiting enzyme of Tau synthesis (Tsuboyama-Kasaoka et al. 2006), and Tau supplementation was prone to prevent obesity in both diet-induced and genetically obese mice supposedly by activating energy expenditure including fatty acid β-oxidation in white adipose tissue.

Recently a randomized double-blind placebo-controlled study reported 8 week Tau supplementation with nutritional counselling increased adiponectin levels and decreased markers of inflammation (high-sensitive C-reactive protein) and lipid peroxidation in obese women without any significant reduction in body weight from the control (Rasa et al. 2014). Since obesity-induced inflammatory reactions cause endothelial dysfunction by reducing nitric oxide (NO) bioavailability through oxidative stress (Iantorno et al. 2014), Tau, known to form Tau chloramine to modulate

oxidative stress resulting from inflammatory reactions (Kim and Cha 2014), is expected to prevent cardiovascular complications caused by obesity, even if Tau's short term effect on obesity is not marked.

This study showed that hypercholesterolemia in the lowest quintile of Tau/Cre is 1.9–2.2 times more than in the highest quintel, with and without the adjustment of confounding variables. Tau supplementation decreases high-fat diet induced hyperlipidemia in stroke-prone SHR (Murakami et al. 1996), hamsters (Murakami et al. 2002b) and Japanese quail (Murakami et al. 2010) supposedly by mechanisms involving conjugation with bile acids and alterations in bile acid synthesis. Tau activates mRNA expression and enzymatic activity of cytochrome P450 7A1 (CYP7A), a rate-limiting enzyme of bile acid synthesis (Murakami et al. 1996; Yokogoshi et al. 1999). Bile acids then conjugate with Tau, which lowers blood TC as observed in the inverse association between Tau and TC, reported by the previous and the present CARDIAC data analyses (Yamori et al. 2009, 2010a, b).

Moreover, Tau exerts anti-atherosclerotic effects in addition to its hypocholesterolemic effects (Murakami 2014). As indicated by an experiment on Watanabe heritable hypercholesterolemic rabbits (Murakami et al. 2002b), Tau prevented atherosclerotic lesions, with a reduction in the marker of lipid peroxidation, but without a significant effect on lipidemia. The mechanism is supposed to be due to the reduction of the major receptor for oxidized low-density lipoprotein (LDL), the lectin-like oxidized LDL receptor by Tau treatment (Gokce et al. 2011).

The present study indicates that increased Tau intake reduces hypertension, obesity and hypercholesterolemia, the major risks of CHD and therefore, contributes to the prevention of CHD, as we first reported the inverse association of 24-h urinary Tau with the mortality rates of CHD (Yamori et al. 1996, 2001).

There are some limitations to our study. First, a cause-effect association cannot be determined from the present analysis because of the cross-sectional study design. Second, we did not examine other related serum biomarkers, such as triglycerides, because of the difficulty of asking the participants to fast for more than 12 h in a worldwide cross-country study. LDL and HDL-cholesterol was not analyzed as well because frozen serum samples had to be sent to a standardized analysis center in accordance with the CARDIAC multicenter study protocol (WHO-CARDIAC Study group 1986, 1990). Apolipoproteins, coronary heart disease risk factors (McQueen et al. 2008), were not analyzed because of the difficulty of setting up a standardized method of analysis at the time of designing the CARDIAC Study in 1985 (WHO-CARDIAC Study group 1986). Our study also lacks data on blood glucose levels and HbA1c, biomarkers of diabetes and key components of the metabolic syndrome.

However, this study also has several strengths. First, the Tau/Cre ratio of the 24-h urine samples varied widely because the CARDIAC Study was a worldwide, multicenter study including participants with various dietary customs, and thus we could detect a significant inverse association between the Tau/Cre ratio and cardiovascular disease risk factors. Second, we assessed the dietary intake of nutrients using the 24-h urine sample, which enabled an objective evaluation of dietary intake.

5 Conclusion

Higher 24-h urinary Tau/Cre ratio was associated with lower cardiovascular disease risk factors, including BMI, BP, TC, obesity, hypertension and hypercholesterolemia. Tau deficiency was related to increased susceptibility to hypertension among the participants in the CARDIAC study, irrespective of ethnic differences, living conditions and gender.

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Use of Taurine During Rehabilitation After Cardiac Surgery

Evgeny Averin

Abbreviations

AH	Arterial hypertension
BID	"Bis in die" twice a day
BP	Blood pressure
CH	Concentric hypertrophy
CHD	Coronary heart disease
CHF	Chronic heart failure
CI	Cardiac index
CR	Concentric remodelling
ECG	Electrocardiogram
EDD	End-diastolic diameters
EDV	End-diastolic volumes
EF	Ejection fraction
EH	Eccentric hypertrophy
ESD	End-systolic diameters
ESV	End-systolic volumes
HDL-C	High-density lipoprotein cholesterol
HR	Heart rate
IVST	Inter-ventricular septum thickness
LDL-C	Low-density lipoprotein cholesterol
LVEF	Left ventricular ejection fraction
LVMM	Left ventricular myocardial mass
LVPW	Left ventricle posterior wall thickness

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NG	Normal geometry
NYHA	New York heart association
RWT	Relative walls thickness
SI	Systolic index
SV	Systolic volume
QoL	Quality of life
TG	Triglyceride
WAM	Well-being, activities and mood

1 Introduction

Presently, Russian cardiac surgery care is experiencing rapid development. A growing number of patients have gained access to high-technology cardiac surgery. Meanwhile, a successful operation is not always a guarantee of a patient's return to his/her working and social activities. In this context, both the success of an operation and the patients' rehabilitation at health resorts and during out-patient period care should be attentively addressed.

Cardiac surgery involving cardiopulmonary bypass leads to impaired myocardial metabolism. Indeed, Suleiman et al. (1993) demonstrated a reduction in taurine levels in myocardial biopsy samples following cardioplegia. The authors suggested the use of Taurine-based drugs both prior, during and after the operation for the purpose of limiting myocardial ischemic damage.

Doddakula et al. (2010) demonstrated the anti-inflammatory and anti-arrhythmic effects of Taurine cardioplegia solutions during post-surgical care.

The explorative study of Sahin et al. (2011) evaluated the role of taurine as an agent for decreasing ischemic cellular damage, presumably due to its antioxidant actions.

In the literature, we could not reveal any studies that have employed taurine during the recovery period after cardiac surgery.

This study aimed to define the influence of taurine on key clinical, instrumental, laboratory and psychological measurements during the rehabilitation period after cardiac surgery.

2 Methods

2.1 Two-Dimensional Transthoracic Echocardiography

The assessment of cardiac morpho-functional parameters included the quantitative 2-dimension heart ultrasound examination with the use of standard technique Lang et al. (2006) using the ultrasonograph ACUSON X128P/10 m (USA) with V4C transducer. The following parameters were measured: linear dimensions of the

cardiac cavities (left atrium and right ventricle anterior-posterior diameters, left ventricle end-systolic (ESD) and end-diastolic diameters (EDD)), left ventricle end-systolic (ESV) and end-diastolic volumes (EDV), left ventricular ejection fraction (LVEF) (using the Teicholz formula [L.E. Teicholz et al.]), inter-ventricular septum thickness (IVST), left ventricle posterior wall thickness (LVPW), left ventricular myocardial mass (LVMM) and LVMM index (using the Devereux formula [Devereux R.B. 1986]).

The type of the left ventricular hypertrophy was determined:

- normal geometry (NG) was recorded in patients with normal values of LVMM (LVMM index, calculated as LVMM to the body area (calculated using the de Bois nomogram) ratio) and the value of the left ventricular relative walls thickness (RWT), calculated with the use of the formula (IVST + LVPW) × 100 %/EDD <45 %
- eccentric hypertrophy (EH)—increased LVMM index, RWT <45 %
- concentric hypertrophy (CH)—increased LVMM index, RWT >45 %
- concentric remodelling (CR) of the left ventricle—normal LVMM index, RWT >45 %.

The following functional parameters were calculated: left ventricular systolic volume (SV), systolic index (SI) calculated as SV to the body area ratio, cardiac index (CI) calculated from the production of SI and heart rate (HR), ejection fraction (EF).

2.2 *The Minnesota Living with Heart Failure Questionnaire*

The Minnesota Living With Heart Failure Questionnaire was used for the evaluation of subjects' quality of life.

This questionnaire included 21 sub-questions. The unique main question was proposed: "Did your heart failure prevent you from living as you wanted during the past month (4 weeks) by…?", with further clarifying sub-questions (Appendix 1). The patient had to mark the answer using the 6-point scale for 0–5. The lowest part of the sheet contained the clarification of the point assignment: 0—no; 1—very little; 2—a little; 3—moderate; 4—much; 5—very much. The patient had to delete the selected score.

The results were processed using the score values. The total value for the whole questionnaire was derived.

2.3 *Well-Being, Activity, Mood Test*

WAM-test was used for the purpose of the operative differential self-assessment of subjects' functional status using three classes of signs: well-being, activity, mood. The subject had to match his/her current state with the range of signs, characterizing

each category, and to assess the intensity of each sign using the 7-point scale, on the special sheet. The mean values of three categories of signs assessed in points were calculated.

2.4 Statistic Analysis

The computerized analysis of the present study was conducted with the use of the package of applied statistical programs SAS (Statistical Analysis System, SAS Institute Inc., USA) using the parametric and non-parametric variation statistics dependent on the measurement scales of each factor.

For the continuous values, the mean values, standard deviations, mean errors, median, interquartile range etc. were calculated. For the nominal values ("yes/no") or the range values, the rate of each ordinal score was calculated in percents.

The analysis of intergroup differences for the continuous values included the calculation of Student's t-tests for independent samples using the corresponding equations, in three different modifications based on the statistical distribution of the specific measures.

In the case of binary factors the significance of difference of the rate of specific factors in two compared groups was assessed with the use of t-test modified with the *arcsin* Fisher transformation.

3 Results

3.1 Design and Basic Information

3.1.1 Materials and Methods

The study involved 48 patients ranging in age from 21 to 62 years who suffer from chronic heart failure (CHF) and have undergone cardiopulmonary bypass surgery. The patients were examined and treated at Volgograd Regional Cardiology Center. Twenty-four patients received the cardiotonic drug Dibicor (Pik-Pharma, Russian Federation), including 18 men (75 %) and 6 women (25 %). The remaining 24 patients composed the control arm.

Exclusion criteria for the study were as follows: diabetes, progressive angina pectoris, uncontrolled CHF, uncontrolled malignant arterial hypertension (AH), symptomatic hepatic and renal failure, anaemia, cancer. The study protocol was approved by the Regional Ethics Committee.

The comparative characteristics of CHF in the active and placebo groups during the post-surgical period are presented in the Table 1.

The taurine group of patients with ischemic CHF consisted of 12 men with coronary heart disease (CHD) undergoing coronary bypass surgery. The age of the

Table 1 Comparative characteristics of study subjects with CHF

Measures	Coronary heart disease		Heart valve defects	
	Taurine	Placebo	Taurine	Placebo
Number of patients	n = 12	n = 12	n = 12	n = 12
Gender				
Male	12 (100 %)	12 (100 %)	6 (50 %)	6 (50 %)
Females	0	0	6 (50 %)	6 (50 %)
Age (years)	55.9 ± 1.4	54.6 ± 1.5	43.5 ± 1.3	42.7 ± 1.5
Disability	5 (41.7 %)	5 (41.7 %)	6 (50 %)	6 (50 %)
Employed	6 (50 %)	6 (50 %)	6 (50 %)	6 (50 %)
Heart failure functional class (NYHA)	1.9 ± 0.1	1.9 ± 0.1	2.0 ± 0.1	2.1 ± 0.1
Arterial hypertension	10 (83.3 %)	10 (83.3 %)	4 (33.3 %)	4 (33.3 %)
Kidney disorder	4 (33.3 %)	4 (33.3 %)	3 (25 %)	3 (25 %)
Myocardial infarction	8 (66.6 %)	8 (66.6 %)	0	0

patients varied from 47 to 62 years (the mean age was 55.9 ± 1.4 years). Half of the patients were employed. Before surgery, the NYHA functional classification of heart failure corresponded to 1.9 ± 0.1. The angina pectoris functional class was assessed as 3.1 ± 0.1, with 66.6 % of the patients having a history of myocardial infarction. The mean number of bypasses implanted was 3.2 ± 0.3. The placebo group of the ischemic CHF patients included 12 men with CHD who underwent coronary bypass surgery. The age of the placebo patients varied from 47 to 62 years (the mean age was 54.6 ± 1.5 years). Half of the patients were employed. Before surgery, the NYHA functional classification of heart failure was 1.9 ± 0.1. The angina pectoris functional class before the surgery was 3.0 ± 0.1, with 66.6 % of the patients having a history of myocardial infarction. In the mean, 3.1 ± 0.3 of bypasses were implanted. The average period between the first diagnosis of CHD and surgery in both study groups was 5.1–5.3 years.

The taurine arm of the CHF study of patients with acquired heart valve defects involved 12 patients undergoing valve replacement surgery. Women constituted half of the patients (six men and six women). The study patients varied from 29 to 61 years of age (the mean age was 43.5 ± 1.3 years, with the mean age of men being 42.1 ± 0.8 and women 45.4 ± 1.2). Half of the patients were employed. The average value of the NYHA functional class of heart failure before surgery was 2.0 ± 0.1. One-valve and two-valve disease were diagnosed in nine patients (75 %) and three patients (25 %), respectively. Twelve CHF patients who underwent surgery due to acquired heart valve disease, entered the placebo group. The numbers of men and women in this group were equal (six men and six women). The study patients were 29–61 years old (the mean group age was 42.7 ± 1.5 years, the mean age of men was 42.0 ± 1.1 and women 44.7 ± 1.7 years). Half of the patients were employed. Before surgery, the average value of the NYHA heart failure functional class was 2.1 ± 0.1. One-valve and two-valve diseases were diagnosed in nine patients (75 %) and in

three patients (25 %), respectively. The mean period between diagnosis and surgery in the two groups of patients with heart valve malformations was 10.7 ± 3.3 and 10.3 ± 3.1 years, respectively.

In general, the number of cases of arterial hypertension was comparable among the CHF patients in the taurine and placebo groups. The two groups of patients included an equal number of cases of postcardiac injury syndrome and post-haemorrhagic anaemia, as well as cases of laryngitis associated with tracheal intubation.

Taurine was administered at a dose of 250 mg BID for a period of 3 months. Treatment started during in-hospital stay, 3 weeks after surgery, at a point in which the state of the patients was adequately stable. The subjects were placed on medication during their sanitarium treatment and in community clinics.

The treatment of CHF was conducted in accordance with the national recommendations (of the Russian Scientific Society of Cardiology and Society of Specialists in Heart Failure) establishing the principles of CHF diagnosis and treatment (National guideline on the diagnosis and treatment of CHF 2003).

The follow-up and examination were conducted at the cardiac surgery hospital (Visit 1), 3 weeks after cardiac surgery (coronary bypass surgery or valve replacement surgery). After being discharged from the hospital, the patients were further treated at the health resort "Volgograd". Following the rehabilitation in a sanitarium, the subjects continued follow-up with cardiologists and general practitioners at their place of residence. Three months after discharge from the hospital, the study subjects completed Visit 2 at the Rehabilitation Department of the Center for Cardiac Surgery.

During Visit 1, patients' status was assessed (Fig. 1).

During Visits 1 and 2, the clinical examination was conducted, including blood pressure (BP) measurement, assessment of subjects' well-being, activities and mood (with the use of WAM-test) and assessment of the quality of life using the Minnesota Living With Heart Failure Questionnaire. A 12-lead electrocardiogram (ECG) was recorded in each subject. Laboratory blood tests were conducted.

Within safety assessment, any adverse events were recorded, as well as the serious adverse events (death, risk of death, events requiring hospitalization or prolonged

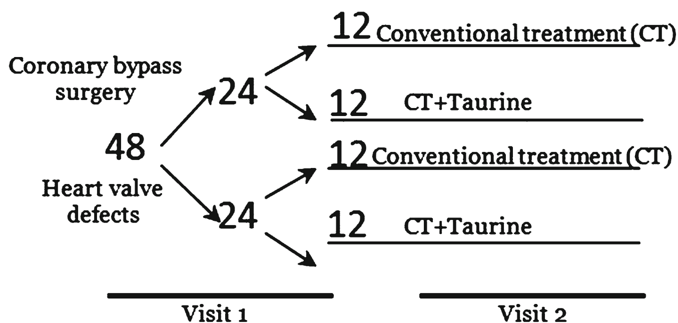

Fig. 1 Subjects' distribution in the study groups

hospitalization, events leading to permanent or significant disability and/or incapacity to work). Adverse events included any negative signs, symptoms or medical conditions (diseases) that developed after the start of treatment with the study drug that was independent of the presence of the study treatment (drug).

3.2 Heart Valve Replacement Surgery Group

Patients with heart valve malformations enrolled into the active treatment and placebo arms had the similar symptoms of CHF. At the baseline, New-York Heart Association (NYHA) class of the heart failure was 2.0 ± 0.1 in the active treatment arm and 2.1 ± 0.1 in the conventional treatment (control) arm. The results of the 6-min walk test did not reveal any significant differences between the active and control arms (362.7 ± 63.4 m vs. 367.3 ± 98.1 m, respectively).

The decrease in NYHA CHF functional class amounted to 16.5 % in the active treatment arm ($p < 0.05$) and to 4.8 % in the control arm.

EF increased by 7.6 % in the active treatment group ($p < 0.05$), compared with 2.4 % in the control group. The inter-ventricular septum thickness was reduced 5.7 % in the taurine group ($p < 0.005$) and by 1.9 % n the control group. Treatment with taurine caused a 10.6 % reduction in inter-ventricular septum thickness ($p < 0.005$) compared with a 2.7 % reduction in the control group. Notably, at the end of the study the left ventricular posterior wall thickness (LVPW) was significantly smaller by 6.5 % ($p < 0.001$) in the control arm (Table 2).

LVMMI decreased by 5.9 % in the active treatment group ($p < 0.05$) compared with 2.9 % in the control group. At the end of the study, LVMMI was significantly lower (by 4.5 %) in the active treatment group compared with the control group ($p < 0.05$).

The level of TG decreased by 12.5 % in the active treatment group ($p < 0.05$) relative to the control group, whose levels remained unchanged. At the end of the study, TG levels were significantly lower (by 12.5 %) in the active treatment group compared with the control group ($p < 0.05$) (Table 3).

3.3 Coronary Bypass Group

The patients undergoing coronary bypass surgery were divided into either the active treatment or the placebo arms; both groups exhibited similar clinical symptoms of CHF. The baseline NYHA heart failure functional class was 1.9 ± 0.1 in both arms. The results prior to treatment for the 6-min walk test did not reveal any differences between the active treatment and control treatment arms (407.7 ± 71.9 m vs. 406.1 ± 73.4 m, respectively).

The percentage decrease of the NYHA CHF functional classification was 15.8 % in the active treatment arm ($p < 0.01$) and 5.3 % in the control arm.

Table 2 Morpho-functional heart measures in patients undergoing valve replacement surgery due to the heart valve defects

Measures	Taurine		Placebo	
	Before treatment	After treatment	Before treatment	After treatment
SV (mL)	77.1 ± 4.5	70.2 ± 4.8*	74.4 ± 4.8	72.8 ± 2.6
EF (%)	57.9 ± 4.3	62.3 ± 5.1[K]	58.8 ± 5.1	60.2 ± 5.0
LVEDD (mm)	52.2 ± 1.1	51.3 ± 1.3	51.9 ± 1.2	51.1 ± 1.1
LVESD (mm)	35.1 ± 1.3	34.4 ± 1.1	34.8 ± 1.2	34.5 ± 1.1
IVST (mm)	10.6 ± 0.4	10.0 ± 0.4*	10.5 ± 0.4	10.3 ± 0.3
LVPW (mm)	11.3 ± 0.4	10.1 ± 0.5[Q]	11.1 ± 0.5	10.8 ± 0.2[F]
Aorta (mm)	36.5 ± 1.0	36.2 ± 1.1	36.6 ± 1.1	36.4 ± 0.5
PA (mm)	23.6 ± 0.7	23.3 ± 0.4	23.7 ± 0.4	23.5 ± 0.5
LA (mm)	47.7 ± 1.4	45.5 ± 1.3*	47.2 ± 1.3	46.7 ± 1.1[H]
RA (mm)	40.0 ± 1.1	38.1 ± 1.1[Y]	39.9 ± 1.1	39.1 ± 1.1[H]
LVMMI (g/m^2)	136.9 ± 6.8	128.8 ± 7.7[K]	138.8 ± 9.2	134.8 ± 4.3[H]

SV systolic volume, *EF* ejection fraction, *FS* percent fractional shortening, *RV* right ventricle, *LVEDD* left ventricle end-diastolic diameter, *LVESD* left ventricle end-systolic diameter, *IVST* inter-ventricular septum thickness, *LVPW* left ventricular posterior wall, *PA* pulmonary artery, *RA* right atrium, *LVMMI* left ventricular myocardial mass index

[K]$p < 0.05$; *$p < 0.005$; [Y]$p < 0.002$; [Q]$p < 0.001$—vs. the baseline level

[H]$p < 0.05$; [Z]$p < 0.005$; [F]$p < 0.001$—vs. patients in Taurine arm

Table 3 Morpho-functional heart measures in patients undergoing valve replacement surgery due to the heart valve defects

Measures	Taurine		Placebo	
	Before treatment	After treatment	Before treatment	After treatment
Total cholesterol (mmol/L)	5.4 ± 1.1	5.2 ± 0.9	5.3 ± 0.8	5.2 ± 0.9
LDL-C (mmol/L)	4.2 ± 0.9	3.9 ± 0.7	4.2 ± 0.8	4.0 ± 0.7
HDL-C (mmol/L)	1.1 ± 0.4	1.2 ± 0.3	1.1 ± 0.4	1.1 ± 0.4
TG (mmol/L)	2.4 ± 0.3	2.1 ± 0.3[K]	2.4 ± 0.5	2.4 ± 0.3[H]

[K]$p < 0.05$—vs. the baseline (before treatment) level

[H]$p < 0.05$—vs. patients in the Taurine group

LVEF increased by 9.0 % in the active treatment group ($p < 0.05$), compared with a 1.5 % increase in the control group. The inter-ventricular septum thickness was reduced by 6.5 % in the taurine group ($p < 0.002$) and by 2.8 % in the control group. The left ventricular posterior wall thickness was reduced by 7.2 % in the taurine group ($p < 0.002$) and by 2.7 % in the control group. Notably, at the end of the study the IVST and LVPW thickness were significantly smaller than those in the control arm; the reduction was 3.8 % ($p < 0.05$) and 4.6 % ($p < 0.05$), respectively. LVMMI was decreased by 5.8 % in the active treatment group ($p < 0.05$), compared with 2.9 % in the control group (Table 4).

Table 4 Morpho-functional heart measures in patients undergoing the coronary bypass surgery

Measures	Taurine		Placebo	
	Before treatment	After treatment	Before treatment	After treatment
SV (mL)	75.3 ± 4.1	73.8 ± 4.3	75.1 ± 4.3	73.5 ± 3.1
EF (%)	51.1 ± 5.3	55.7 ± 5.2^{K}	52.1 ± 5.2	52.9 ± 5.1
LVEDD (mm)	50.8 ± 1.1	50.9 ± 1.1	50.9 ± 1.2	51.1 ± 1.1
LVESD (mm)	33.8 ± 1.3	33.4 ± 1.1	34.0 ± 1.2	33.8 ± 1.1
IVST (mm)	10.8 ± 0.4	10.1 ± 0.4^{Y}	10.8 ± 0.4	10.5 ± 0.3^{H}
LVPW (mm)	11.1 ± 0.4	10.3 ± 0.5^{Y}	11.1 ± 0.5	10.8 ± 0.2^{H}
Aorta (mm)	33.8 ± 1.0	34.1 ± 1.1	33.6 ± 1.1	34.0 ± 0.5
PA (mm)	20.6 ± 0.7	20.3 ± 0.4	20.7 ± 0.4	20.5 ± 0.5
LA (mm)	41.2 ± 1.3	40.5 ± 1.3	41.1 ± 1.3	40.6 ± 1.1
RA (mm)	34.3 ± 1.1	34.1 ± 1.1	34.4 ± 1.1	34.2 ± 1.1
LVMMI (g/m^2)	137.1 ± 8.7	129.1 ± 8.7^{K}	138.1 ± 9.1	134.1 ± 7.1

$^{K}p<0.05$; $^{Y}p<0.002$—vs. the baseline (before treatment) level
$^{H}p<0.05$—vs. patients in the Taurine group

Table 5 Morpho-functional heart measures in patients undergoing the coronary bypass surgery

Measures	Taurine		Placebo	
	Before treatment	After treatment	Before treatment	After treatment
Total cholesterol (mmol/L)	4.9 ± 0.9	4.6 ± 0.8	4.9 ± 0.8	4.7 ± 0.9
LDL-C (mmol/L)	3.6 ± 0.6	3.4 ± 0.6	3.6 ± 0.8	3.5 ± 0.7
LDL-C (mmol/L)	1.1 ± 0.3	1.2 ± 0.3	1.1 ± 0.4	1.1 ± 0.4
TG (mmol/L)	2.2 ± 0.3	1.9 ± 0.3^{K}	2.4 ± 0.5	2.3 ± 0.3^{H}

$^{K}p<0.05$—vs. the baseline (pre-treatment) level
$^{H}p<0.05$—vs. patients in the Taurine group

The level of TG decreased by 13.6 % in the active treatment group ($p<0.05$), compared to a decrease by 4.2 % in the control group. At the end of the study, TG levels were significantly lower (by 17.4 %) in the active treatment group than the control group ($p<0.05$) (Table 5).

3.4 *The Influence of Taurine on the Quality of Life and Psychological Status of Patients with CHF, Undergoing the Cardiac Surgery with Cardiopulmonary Bypass*

The general improvement of the quality of life (QoL) in patients with CHF was 22.6 % on average ($p<0.05$), vs. 16.6 % in the placebo arm. Meanwhile, QoL improved by 17.7 % among male subjects and by 27.7 % among women ($p<0.05$).

Table 6 Time-related changes of the Quality of Life in patients with CHF in the Taurine arm

	Quality of Life, score			
	Taurine		Placebo	
Name	Before treatment	After treatment	Before treatment	After treatment
Group mean	34.1 ± 2.3	26.4 ± 2.5*	33.7 ± 2.1	28.1 ± 2.4
Males	31.6 ± 4.9	26.0 ± 5.9	31.3 ± 4.6	27.0 ± 5.4
Females	38.2 ± 5.0**	27.6 ± 5.2*	37.2 ± 5.0	31.7 ± 5.2
Heart valve defects group				
Group mean	35.1 ± 2.3	26.9 ± 2.5*	35.3 ± 2.3	29.5 ± 2.5
Males	28.1 ± 2.7	24.3 ± 3.1	28.3 ± 2.6	26.1 ± 2.9
Females	38.2 ± 5.0	27.6 ± 5.2*	37.2 ± 5.0	31.7 ± 5.2
Coronary bypass group				
Group mean (men)	33.1 ± 2.4	24.9 ± 2.5*	32.6 ± 2.4	27.3 ± 2.5

*$p < 0.05$ vs. pre-treatment values
**$p < 0.05$ vs. male subjects

In the placebo arm of patients with CHF the changes were not significant (13.7 and 14.8 %), in line with little improvement in CHF. The time-related changes in the quality of life in the taurine arm of CHF patients are presented in the Table 6.

QoL improvement in the valve defect group was 23.5 % ($p < 0.05$) vs. 16.4 % in the placebo arm. In the taurine arm of CHD patients, QoL improved 24.8 % ($p < 0.05$) vs. 16.3 % in the placebo arm. Among male patients, QoL improved 13.5 % vs. 22.8 % among women ($p < 0.05$), while in the placebo group the respective values were 7.8 % and 18.1 %.

Thus, 3-month taurine treatment led to 22.6 % improvement in the quality of life ($p < 0.05$). Male patients in the heart valve defect group had a better baseline quality of life value compared with women. However, they demonstrated a smaller increase in QoL: 13.5 % vs. 22.8 %, respectively.

3.5 Time-Related Changes of the Results of "Well-Being, Activity, Mood" Test

At the end of taurine treatment, the "Well-Being" score of the WAM-test demonstrated a 10.6 % improvement of the group average ($p < 0.05$) vs. 6.4 % in the placebo group. The analysis of gender subgroups revealed larger improvement of the Well-Being factor in women: 12.8 % ($p < 0.05$). In male patients, the growth of this value was not significant (6.5 %). In the placebo arm, the corresponding changes were 8.5 % and 4.3 %, respectively (Table 7).

At the end of taurine treatment, the group mean score of well-being among the patients with heart valve defects had increased 6.5 % ($p < 0.05$) vs. 4.3 % in the placebo arm. The improvement in well-being rose 12.8 % among women and 6.5 % among men in the taurine arm, but only 8.5 % and 2.2 %, respectively, in the placebo arm.

Table 7 The Well-Being score of WAM-test in CHF patients enrolled into the taurine arm

Name	Well-Being, score			
	Before taurine treatment	After taurine treatment	Before placebo treatment	After placebo treatment
Group mean	4.7±0.1	5.2±0.2*	4.7±0.1	5.0±0.2
Males	4.6±0.2	4.9±0.2	4.6±0.2	4.8±0.2
Females	4.7±0.2	5.3±0.1*	4.7±0.2	5.1±0.1
Heart valve defects group				
Group mean	4.7±0.1	5.0±0.1*	4.7±0.1	4.9±0.2
Males	4.6±0.1	4.9±0.2	4.6±0.1	4.7±0.2
Females	4.7±0.2	5.3±0.1*	4.7±0.2	5.1±0.1
Coronary bypass group				
Group mean (men)	4.6±0.1	5.2±0.1*	4.7±0.1	5.1±0.2

*$p<0.05$ vs. pre-treatment values

Table 8 The activity score of WAM-test in CHF patients enrolled into the taurine arm

Name	Activity, score			
	Before taurine treatment	After taurine treatment	Before placebo treatment	After placebo treatment
Group mean	4.6±0.1	5.1±0.1*	4.6±0.1	5.0±0.2
Males	4.6±0.2	5.1±0.2*	4.6±0.2	4.9±0.2
Females	4.7±0.1	5.2±0.1*	4.7±0.1	5.1±0.2
Heart valve defects group				
Group mean	4.7±0.1	5.2±0.1*	4.7±0.1	5.1±0.1
Males	4.6±0.1	5.1±0.1*	4.6±0.1	5.0±0.1
Females	4.7±0.1	5.2±0.1*	4.7±0.1	5.1±0.2
Coronary bypass group				
Group mean (men)	4.5±0.1	5.0±0.1*	4.5±0.1	4.9±0.2

*$p<0.05$ vs. pre-treatment values

The baseline level of well-being in patients undergoing coronary bypass surgery was similar to the patients with the heart valve defects. However, the former group demonstrated higher (6.1 %) improvement of well-being following taurine treatment ($p<0.05$).

The subjects enrolled in the taurine arm demonstrated a 10.9 % ($p<0.05$) increase 1 in the "Activity" score compared with 8.7 % in the placebo arm. Among women, this value was 2.0 % higher than in men both before and after taurine treatment, with the mean value being 10.6 %. The increase in the score in the placebo arm was 8.5 % (Table 8).

The analysis of diagnosis-based subgroups showed higher "Activity" scores in patients with heart valve defects both before and after taurine treatment (4.4 %), compared to the CHD group; the post-treatment increase in the taurine arm was 11.1 %.

Table 9 The Mood score of WAM-test in CHF patients enrolled into the taurine arm

Name	Mood, score			
	Before taurine treatment	After taurine treatment	Before placebo treatment	After placebo treatment
Group mean	5.2±0.1	5.6±0.1*	5.2±0.1	5.4±0.1
Males	5.3±0.2	5.6±0.1	5.3±0.2	5.5±0.1
Females	5.1±0.1	5.5±0.2*	5.1±0.1	5.3±0.2
Heart valve defects group				
Group mean	5.3±0.1	5.7±0.1*	5.3±0.1	5.6±0.1
Males	5.3±0.2	5.7±0.1	5.4±0.2	5.7±0.1
Females	5.1±0.1	5.5±0.2*	5.1±0.1	5.3±0.2
Coronary bypass group				
Group mean (men)	5.1±0.2	5.5±0.2	5.1±0.2	5.3±0.2

*$p<0.05$ vs. pre-treatment values

By comparison, in the placebo arm the difference between the diagnosis subgroups was 4.4 % during pre-treatment and 4.1 % after the treatment.

The mean group level of the "Mood" parameter in CHF patients increased by 7.7 % following the 3-month taurine treatment, but only 3.8 % in the placebo arm. Mood improvement in the taurine arm was 7.8 % among women ($p<0.05$) and 5.7 % among men, while it was 3.8 % and 3.9 %, respectively, in the placebo arm (Table 9).

The increase in the "Mood" parameter was 7.8 % among CHF patients in the taurine arm after coronary bypass surgery, but only 3.9 % in the placebo group. The patients with heart valve defects demonstrated a 3.9 % higher value of activity compared to CHD patients, both before and after taurine therapy.

Thus, the general trend of each parameter of WAM-test demonstrates significant improvement. Following taurine treatment, the patients with CHF showed a greater improvement in well-being compared to patients undergoing heart valve surgery without taurine treatment. Moreover, male patients demonstrated a greater improvement in well-being than women. However, the level of activity was higher in female patients. Among the patients with heart valve defects, the activity and mood scores were higher in CHD patients both before and after taurine treatment.

4 Conclusion

1. In patients undergoing heart valve replacement and coronary bypass surgery, the use of taurine resulted in a significant increase in left ventricular ejection fraction and a reduction in left ventricular myocardial mass index and triglyceride levels.

2. Both taurine treatment groups demonstrated a significant improvement in the quality of life.
3. The results of "WAM" testing demonstrated an improvement in Well-being, Activity and Mood in patients enrolled in the taurine arm.

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Newer Insights into the Taurinuria of Vitamin D Deficiency: A Review

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Abbreviations

BBMV	Brush border membrane vesicles
HC	High calcium
LC	Low calcium
PTH	Parathyroid hormone
RXR	Retinoic acid receptor
VDR	Vitamin D receptor
VDRE	Vitamin D receptor element
VLC	Very low calcium
VLP	Very low phosphate

1 Introduction

One role of the kidney is the reabsorption of organic solutes, including amino acids. This process makes sense because amino acids are needed for protein synthesis, both in the kidney and throughout the body. Taurine, needed for its osmolytic and antioxidant properties (Han and Chesney 2010), is conserved by renal tubular reabsorption. Excessive excretion of taurine into the urine is found in several clinical situations, including renal tubular damage, Mendelian disorders such as the Fanconi

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syndrome, renal immaturity, and following a high dietary taurine load (Han and Chesney 2012). Among those states in which excess taurinuria is evident is vitamin D deficiency. Our laboratory has investigated the taurinuria of vitamin D deficiency over the past 30 years. This review serves as a recap of information gleaned from a series of studies in which animals were made vitamin D-deficient, their urinary excretion of taurine was measured and the uptake of taurine by isolated renal brush border membrane vesicles (BBMV) was evaluated. Further studies were performed in renal cells in culture, including reporter assays, taurine uptake measurements and taurine transporter protein abundance.

One of the causes of excessive taurinuria is vitamin D deficiency (Dabbagh et al. 1989a), probably as a component of the generalized aminoaciduria of that pathophysiologic state (Chisolm 1959; Hassanein and Patel 1967; Jonxis and Huisman 1953). Taurinuria (and aminoaciduria) were initially felt to be due to the secondary hyperparathyroidism that accompanies the vitamin D-deficient state (Chesney and Harrison 1975; Fraser et al. 1967; Harrison 1959; Scriver et al. 1964). The concept was that parathyroid hormone (PTH) directly diminished renal tubular reabsorption of amino acids. This seemed a plausible hypothesis because (1) PTH values were elevated due to hypocalcemia, and (2) repletion with vitamin D corrected both elevated PTH values and generalized aminoaciduria (Grose and Scriver 1968; Phillips et al. 1980). Another reason that excessive aminoaciduria was ascribed to PTH action is that the hormone has direct physiologic action in the kidney and results in phosphaturia and enhanced calcium reabsorption (Scriver 1974). Furthermore, parathyroid extract injection enhanced the excretion of phosphate and amino acids in parathyroidectomized vitamin D-deficient rats (Grose and Scriver 1968).

A number of observations led to a shift in thinking to posit that vitamin D (and its metabolites), per se, influenced amino acid reabsorption. Among these were clinical observations that included the finding of aminoaciduria in subjects with nonossifying fibromata, in which PTH and 1,25$(OH)_2$D values are low (Drezner and Feinglos 1977); the finding of aminoaciduria in only a minority of patients with primary hyperparathyroidism (Cusworth et al. 1972); and persistence of aminoaciduria despite correction of hypophosphatemia in vitamin D-deficient children (Brodehl et al. 1971; Weber et al. 1973). Some cases of renal Fanconi syndrome and a case of tumor-associated osteomalacia with low or absent circulating 1,25$(OH)_2$ vitamin D, normal serum PTH and aminoaciduria were described as well (Phillips 1980). When patients with chronic kidney disease, who had both secondary hyperparathyroidism and impaired renal synthesis of 1,25$(OH)_2$ vitamin D were treated with vitamin D metabolites, it was found that reversal of aminoaciduria did not correlate with changes in PTH, but rather with the extent of vitamin D replacement, especially as regards to 1,25$(OH)_2$ vitamin D values (Phillips et al. 1980).

These anomalies, and interesting clinical situations suggesting that other factors are at play, led us to examine the role of vitamin D status in aminoaciduria, in general, and taurinuria, in particular. We accordingly performed studies *in vivo* in rats as well as in isolated proximal tubule BBMV and in cultured cells. These sequential studies led to newer insights regarding the mechanism of taurinuria in vitamin D deficiency.

2 Methods

2.1 Animals and Diets

Weanling Sprague-Dawley rats (Holtzman Lab, Madison, WI) were used. Dams had been fed a diet containing only 0.2 IU of vitamin D_2/g chow rather than the usual 4.4 IU/g, and hence pups were vitamin D deficient at birth. Animals were housed in an internal room with no access to ultraviolet rays. Only tungsten lamps were used, which do not emit light in the ultraviolet spectra.

Animals were fed one of five diets listed in Table 1, and all studies were performed after 4–6 weeks. One group, chosen to demonstrate the influence of acute supplementation with $1,25(OH)_2D_3$, was fed a very low calcium (VLC) diet and injected once daily with 500 pmol $1,25(OH)_2D_3$ for 2 days prior to sacrifice.

On the day prior to sacrifice, urine was collected using metabolic cages. Upon sacrifice, plasma, urine and renal cortex tissue were obtained and stored at −20 °C. Renal cortex was also used to prepare brush border membrane vesicles for taurine uptake studies.

Analyses of protein-free extracts of plasma and urine for taurine and other amino acids, calcium, phosphorus, creatinine and vitamin D metabolites were performed as described (Dabbagh et al. 1989b). PTH values were assessed using a specific rat antibody assay of Chanard et al. (1977).

Renal cortical brush border membrane vesicles were prepared by differentiated centrifugation and $MgCl_2$ precipitation as described in Dabbagh et al. (1991). Purity of the preparation was evaluated by enrichment or reduction of brush border membrane-bound enzymes (γ-glutamyl transpeptidase, sodium-potassium-ATPase, *N*-acetyl-β-D-glucosaminidase, succinic cytochrome c reductase).

Amino acid uptake studies were performed by the rapid Millipore filtration technique at 23 °C for 0.5–45 min. Incubation was begun by the addition of a medium containing unlabelled (cold) and radiolabelled (^3H) taurine. Samples were pipetted onto prewetted 50 μm filters under vacuum and washed three times with 3.0 ml iced stop solution. Filters were counted in a liquid scintillation counter after drying overnight.

Table 1 Rat diets

Type	Protein[a] (%)	Carbohydrate (%)	Mineral content (%)	
			Calcium	Phosphate
Rat chow (normal)	18	62	1.15	0.7
Vitamin D-deficient				
Very low Ca^{2+}	18	62	0.02	0.3
Low Ca^{2+}	18	62	0.47	0.3
High Ca^{2+}	18	62	2.5	0.3
Low PO_4^{3-}	18	62	1.2	0.1

[a]Protein source was casein with cysteine and methionine content of 0.3 and 0.6 % respectively. Adapted from Dabbagh et al., *Miner Electrolyte Metab* 1989; 15:221–232

Because PTH exposure results in an increase in intracellular cAMP in proximal tubule cells and in urinary cAMP, we sought to determine whether cAMP influenced amino acid uptake. We evaluated the effect of a more stable analog, dibutyryl cAMP, on taurine uptake by rat renal BBMV as described (Dabbagh et al. 1989c). Uptake of ^{32}P prepared in 50 μM phosphate was also examined, as PTH exposure results in phosphaturia.

2.2 Cells

LLC-PK1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum and 50 units/mL penicillin, and incubated at 37 ° C in 5 % CO_2. Cells were fed every 3 days. Taurine transport studies were performed on confluent monolayers using Earl's balanced salt solution to which 50 μM cold and ^{14}C-labelled taurine was added. Cells were incubated for 30 min; uptake was terminated by removal of uptake buffer. Cells were dissolved in 1 % SDS in 0.2 N NaOH and radioactivity was counted in a liquid scintillation analyzer.

Cells were also used for Western blot analysis. Cell membranes were incubated with primary antibodies for 1 h at room temperature. Blots were washed with buffer and incubated with horseradish peroxidase-linked secondary antibody for a subsequent hour and then proteins of interest were detected using a chemiluminescence kit.

2.3 Statistical Analysis

Data comparisons were made using *t*-tests, analysis of variance and linear regression using the SPSS statistical package.

3 Results

3.1 Animal Studies

A significant aminoaciduria (of 10 amino acids, including taurine) was found in all vitamin D-deficient groups as compared to rats fed the control chow (Dabbagh et al. 1989b). Acute administration of $1,25(OH)_2D$ did not reverse aminoaciduria *in vivo* (Fig. 1). The effect of vitamin D deficiency on plasma, renal tissue and urinary taurine values is shown in Table 2. Serum 25(OH)D and $1,25(OH)_2D$ values were significantly reduced in all vitamin D-deficient groups, but not in control or acutely treated animals (Table 3). Serum calcium was significantly ($p<0.01$) lower in all vitamin D-deficient groups (except in the animals fed the low phosphate diet) as

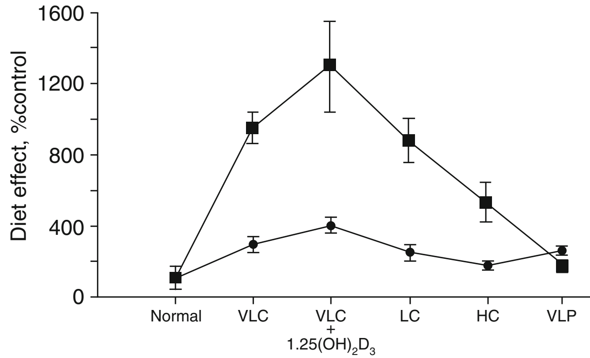

Fig. 1 The effect of diet on plasma PTH (*squares*) and total urinary amino acid (*circles*) excretion. Values represent mean ± SEM. Reproduced with permission from Dabbagh et al., *Miner Electrolyte Metab* 1989; 15:221–232

Table 2 Effect of vitamin D deficiency on plasma, renal tissue and urinary taurine values in rats

Diet	Plasma (μmol/L)	Renal cortex (μmol/g)	Urine (μmol/mg Cr)
Normal chow	16.58 ± 1.54 n = 7	4.22 ± 1.04 n = 4	5.92 ± 1.16 n = 7
Vitamin D-deficient			
Very low Ca^{2+}	19.33 ± 2.21 n = 7	1.3 ± 0.03 n = 3	18.73 ± 1.66 n = 6 $p < 0.001$
Very low Ca^{2+} + 1,25(OH)2D3	18.00 ± 1.15 n = 6	3.13 ± 0.73 n = 3	26.44 ± 4.26 n = 6 $p < 0.001$
Low Ca^{2+}	16.33 ± 1.38 n = 7	3.74 ± 0.20 n = 2	14.52 ± 2.71 n = 6 $p < 0.02$
High Ca^{2+}	16.25 ± 1.07 n = 4	4.11 ± 0.30 n = 3	9.58 ± 1.15 n = 7 $p < 0.05$
Low PO_4^{3-}	13.56 ± 1.44 n = 7	2.93 ± 0.34 n = 4	13.74 ± 1.84 n = 8 $p < 0.01$

Adapted from Dabbagh et al., *Miner Electrolyte Metab* 1989; 15:221–232

compared to the control group. Serum phosphate was higher in vitamin D-deficient rats fed very low calcium and low calcium diets ($p < 0.01$) and lower in those fed high calcium and low phosphate diets. Plasma PTH was markedly elevated in all deficient groups, except for the low phosphate diet group, as compared to controls (Fig. 2). In the low phosphate vitamin D-deficient group, the plasma PTH level was similar to that in the control group. This is to be expected because phosphate depletion does not stimulate PTH synthesis and secretion.

The excretion of urinary cAMP, an indicator of PTH activity, was high in the very low, low, and high calcium-fed groups, but not in the low phosphate diet

Table 3 Plasma concentrations of $25(OH)D_3$ and $1,25(OH)_2D_3$ in animals fed the various diets

	$25(OH)D_3$ (ng/mL)	$1,25(OH)_2D_3$ (pg/mL)
Normal	52.01 ± 8.27	31.9825 ± 3.93
VLC	4.88 ± 1.36**	1.87 ± 1.02**
VLC + $1,25(OH)_2D_3$	9.7 ± 1.04**	97.58 ± 21.23*
LC	6.7 ± 0.44**	0.67 ± 0.42**
HC	6.88 ± 0.68**	3.9 ± 1.78**
VLP	6.72 ± 0.68**	1.66 ± 0.79**

Values are mean ± SE of 4–11 determinations
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VLC very low calcium, *LC* low calcium, *HC* high calcium, *VLP* very low phosphate
*$p < 0.01$; **$p < 0.001$ versus control

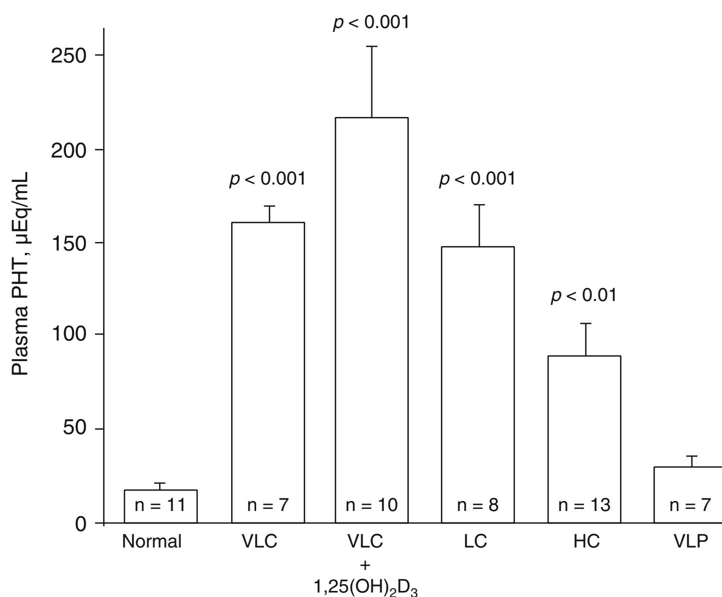

Fig. 2 The effect of vitamin D deficiency on plasma PTH levels. All results are mean ± SEM. Reproduced with permission from Dabbagh et al., *Miner Electrolyte Metab* 1989; 15:221–232

animals, again an expected finding (Fig. 3). While dietary calcium and phosphate influence plasma and urinary calcium and phosphate (data not shown), this study does not support a linkage between secondary hyperparathyroidism and aminoaciduria.

Studies in renal BBMV indicated that the aminoaciduria of vitamin D deficiency is expressed by changes in the brush border membrane, where the TauT transport protein is located. Specifically, in BBMV from animals where taurinuria (and proteinuria) were evident, there was a 21–25 % attenuation in the peak of the overshoot

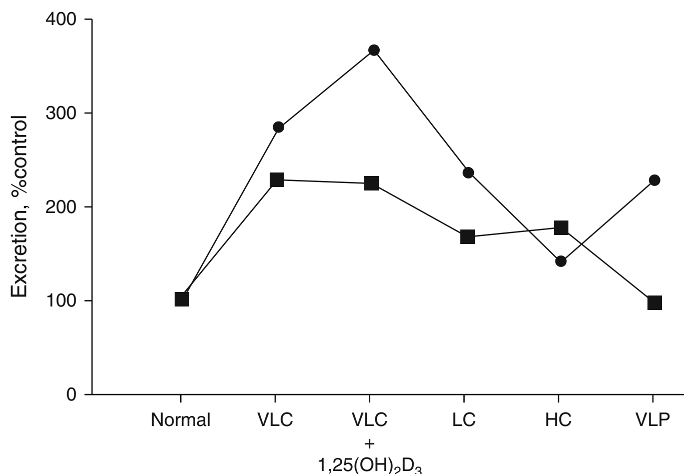

Fig. 3 The extent of the increase in urinary excretion of cAMP (*squares*) and total amino acids (*circles*) in relation to the different diets. Reproduced with permission from Dabbagh et al., *Miner Electrolyte Metab* 1989; 15:221–232

of the sodium-dependent uptake of taurine as compared to taurine uptake by BBMV from rats fed a normal chow diet ($p<0.01$). Acute supplementation with 1,25$(OH)_2D_3$ tended to normalize the V_{max} of taurine uptake (Fig. 4). These studies led to the conclusion that the aminoaciduria associated with vitamin D deficiency manifests at the apical membrane of the proximal tubule. These changes—a diminished overshoot and decrease in V_{max}—occur whether PTH activity is high (low calcium diet groups) or low (phosphate-depleted diet).

Dabbagh et al. (1989c) then examined the role of cAMP and found that incubation of BBMV with dibutyryl cAMP at concentrations between 10^{-4} and 10^{-7} M had no influence on taurine uptake by BBMV. Lysis of vesicles to allow cAMP to be internalized, followed by resealing vesicles, also did not alter taurine uptake. Furthermore, in BBMV prepared from adult rats fed a normal diet, exposure to cAMP and ATP at an intravesicular site did not alter taurine uptake, but the vesicular uptake of ^{32}P was significantly decreased by 24 % (Dabbagh et al. 1989c).

Thus, by inference, PTH, which would increase intracellular cAMP, did not alter taurine uptake, but significantly diminished the 30-, 60-, and 240-s uptake of 50 μM PO_4.

The results indicating an alteration in taurine transport by apical membranes led us to examine how vitamin D might influence the synthesis of the taurine transporter protein using a proximal tubule cell line from porcine kidney (LLC-PK1). We sought to determine if 1,25$(OH)_2D_3$ could alter cellular uptake of taurine. Studies were also performed in 293 cells (human embryonic kidney) and MDCK cells (canine distal tubule). When cells were transfected with a full length *TauT* promoter-reporter gene and co-cultured with 10 μM 1,25$(OH)_2D_3$, there was no change in the activity of the *TauT* promoter (Fig. 5). However, because the vitamin D receptor

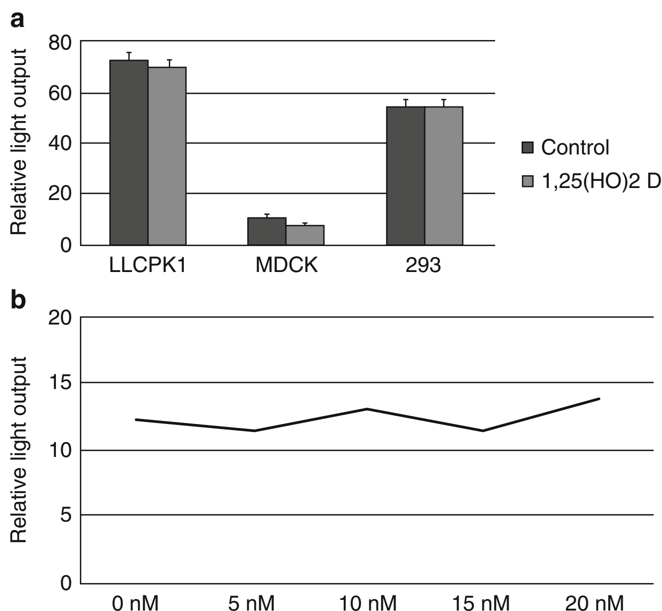

Fig. 4 Effect of 1,25$(OH)_2D_3$ and retinoic acid on *TauT* expression in renal cells. (**a**) Reporter gene assay in LLC-PK1, MDCK and 293 cells; (**b**) Reporter gene assay in LLC-PK1 cells with different doses of vitamin D

(VDR) is part of the retinoic acid receptor (RXR) superfamily of receptors, we examined the interplay between 1,25$(OH)_2D_3$ and all *trans*-retinoic acid relative to *TauT* expression. When cells were incubated with both agents, there was an increase in *TauT* activity, in taurine uptake by cells, and TauT abundance by Western blot analysis of LLC-PK1 cells treated with 1,25$(OH)_2D_3$ and retinoic acid (Fig. 6). Deletion of a regulatory region of the *TauT* promoter, containing several transcription factors and including two AP1 sites, abrogated the influence of retinoic acid/1,25$(OH)_2D_3$ on *TauT* expression. These findings led us to speculate that a synergistic regulation of *TauT* expression may require the formation of the RXR/VDR complex, which in turn binds to the *TauT* promoter region to regulate TauT abundance in LLC-PK1 cells (Fig. 7) (Chesney and Han 2013).

4 Discussion

The finding of taurine in the urine of patients with renal tubular defects or as a component of hereditary aminoacidurias became evident after the development of ion-exchange chromatography (Cusworth et al. 1972). Taurinuria has also been described in patients with Lowe syndrome (Gardner and Brown 1976), in animal

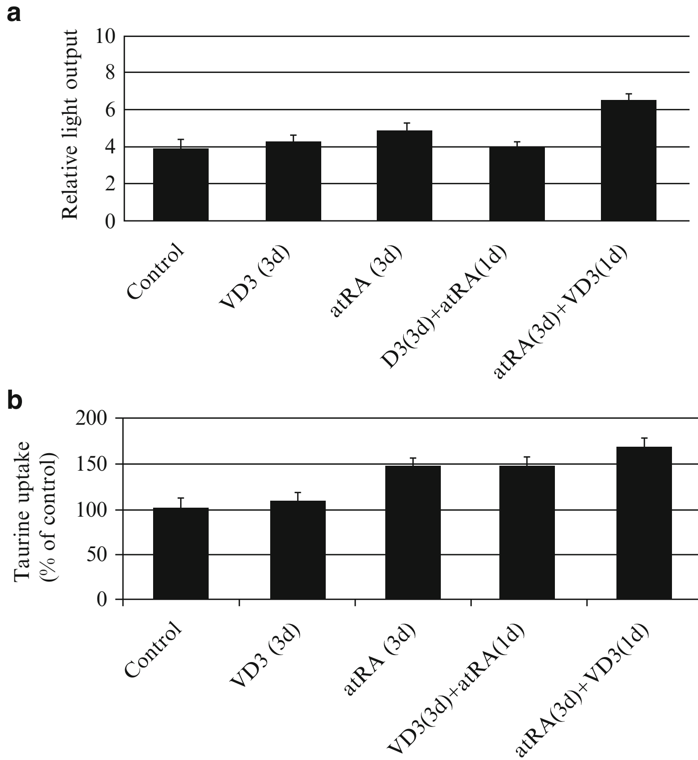

Fig. 5 Upregulation of TauT expression by $1,25(OH)_2D_3$ requires retinoic acid as a co-factor. The combination of vitamin D and all-*trans* retinoic acid (**a**) stimulates *TauT* promoter activity, and (**b**) increases taurine uptake

models of Dent disease (Wang et al. 2000), a monogenic tubular disorder with glycosuria, low molecular weight proteinuria and, at times, in rickets. Taurinuria is a component of generalized aminoaciduria. Many of these conditions are associated with abnormalities of vitamin D metabolism and low 25(OH)D values.

The findings of elevated PTH and aminoaciduria in vitamin D deficiency led numerous investigators to suggest a linkage (Chesney and Harrison 1975; Fraser et al. 1967; Harrison 1959; Hassanein and Patel 1967; Scriver et al. 1964). This review examines studies that discount the role of PTH as a causative factor in the taurinuria and generalized aminoaciduria of vitamin D deficiency.

The studies of Dabbagh et al. (1989a) revealed that aminoaciduria and taurinuria were evident despite the variation in concentrations of PTH in rats on differing vitamin D-deficient diets. In the low phosphate dietary state, there is no signal for increasing PTH secretion, and, accordingly, PTH values are normal, yet these animals have taurinuria. PTH increases urinary cAMP content and this is important in PTH-induced phosphaturia. Taurinuria is also evident despite a marked variability

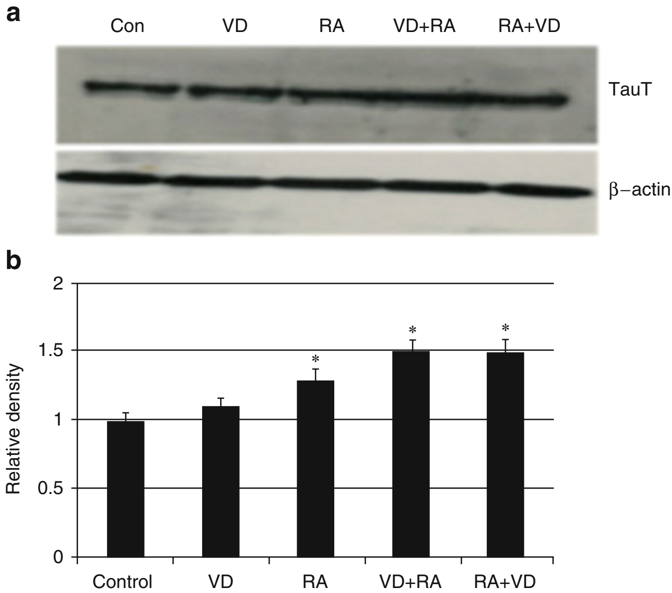

Fig. 6 Effect of $1,25(OH)_2D_3$ and retinoic acid on TauT expression in renal cells. (**a**) Western blot analysis of TauT expression; (**b**) Relative density of Western blot

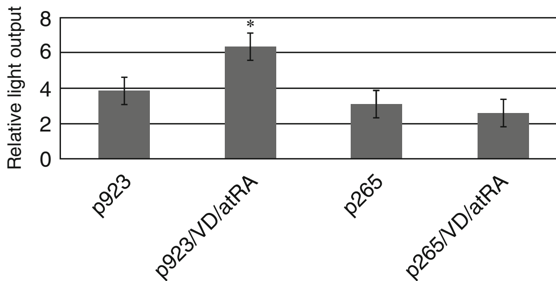

Fig. 7 The VDR/RXR consensus site is located in the proximal promoter region of *TauT* 5′-UTR (untranslated region). *TauT* promoter reporter constructs p923 or p265 were transfected into LLC-PK1 cells and the effect of vitamin D_3 and all-*trans* retinoic acid on *TauT* promoter activity was measured. The promoter activity of construct p923 was increased by 1.5-fold. Construct p265, containing only a basal promoter sequence of *TauT* 5′-UTR, did not show a response

in urinary cAMP values. This study strongly suggests that PTH, per se, and its metabolic product, cAMP, is not responsible for the generalized aminoaciduria of vitamin D deficiency.

The finding that isolated BBMV from rats on the various diets demonstrate a reduction in Na-dependent taurine uptake indicate that the effect is found in the apical membrane, per se. This is the site of active taurine uptake. The change in uptake is expressed as a significant reduction in V_{max}, as well as in the height of the uptake overshoot (Dabbagh et al. 1990). These BBMV, because of extensive

washing, are devoid of metabolites, but are the location of the TauT transporter protein. Even when BBMV are incubated with dibutyryl cAMP, there is no change in taurine uptake (Dabbagh et al. 1989c). These findings are further supported by the partial restoration of the height of the overshoot and restoration of a normal V_{max} in vesicles isolated from vitamin D-deficient rats acutely treated with $1{,}25(OH)_2D_3$.

More importantly, patients with vitamin D-dependent rickets type II, caused by mutations in the vitamin D receptor gene (VDR), have taurinuria and generalized aminoaciduria (Shafeghati et al. 2008). Ironically, these patients have extremely high circulating values of $1{,}25(OH)_2D_3$ that cannot bind to the mutated VDR, but the stimuli for $1{,}25(OH)_2D_3$ synthesis, namely hypocalcemia and elevated PTH, prevail in these patients. This supports a role for the VDR as contributing to aminoaciduria.

An important mechanistic consideration is that $1{,}25(OH)_2D_3$ influences the function of the *TauT* gene following its binding to VDR and further interaction with the vitamin D response element (VDRE) on the promoter region of *TauT*. The study of Chesney and Han (2013) shows that $1{,}25(OH)_2D_3$ alone has no genomic action, but that in conjunction with retinoic acid there is increased *TauT* activity. This is evident from increased gene activity (reporter assay), increased TauT protein (Western blot) and increased taurine uptake by LLC-PK1 cells.

These findings strongly suggest that the aminoaciduria of vitamin D deficiency relates to a lack of up-regulation of taurine transporter synthesis and transfer into the brush border membrane. In this model, replacement of vitamin D to vitamin D-deficient subjects, in the presence of adequate vitamin A stores, will reverse taurinuria, and possibly other aminoacids excessively excreted, although we did not directly examine this.

There exist numerous biologic processes that are regulated by vitamin D acting via its receptor (VDR) and interacting with the VDRE (Fig. 8) (Carlberg and Seuter 2009; Carlberg et al. 2013). The vitamin D receptor is a member of a nuclear receptor superfamily, which also includes other nuclear hormones such as retinoic acid, thyroid hormone, estradiol, progesterone, testosterone, cortisol and aldosterone (Carlberg 1995). While the classical biological role of $1{,}25(OH)_2D_3$ is the regulation of calcium and phosphate homeostasis, transcriptosome-wide analysis indicates that in each cell with a VDR, between 200 and 600 genes are primary targets of vitamin D (Ramagopalan et al. 2010). An example is the finding of a vitamin D response element in the human insulin receptor gene promoter (Maestro et al. 2003). Because vitamin D is involved in so many biologic processes, the finding of a VDRE in the *TauT* promoter is not surprising and has been recently shown (Chesney and Han 2013).

5 Conclusion

In conclusion, insufficient amounts of $1{,}25(OH)_2D_3$ result in taurinuria as part of the generalized aminoaciduria of the deficient state. A defect in taurine uptake is found in proximal tubule cells, which is equivalent to reduced reabsorption from the

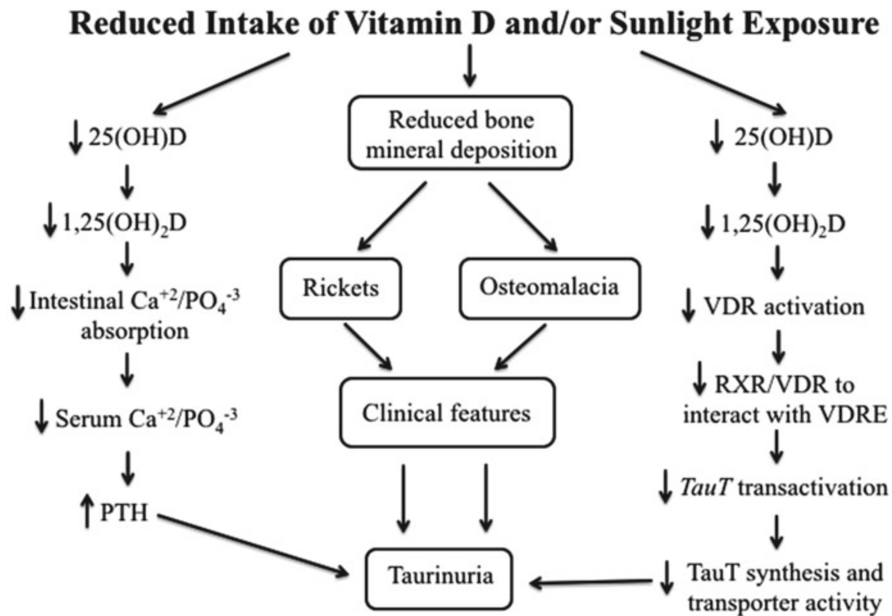

Fig. 8 Mechanisms of aminoaciduria and taurinuria occurring in vitamin D deficiency. *VDR* vitamin D receptor, *RXR* retinoic acid receptor, *VDRE* vitamin D receptor element

glomerular filtrate in the proximal tubule lumen. The studies of Dabbagh et al. reveal that the defect in taurine accumulation is located in the brush border membrane, where sodium-dependent taurine uptake occurs (Han and Chesney 2012) and where the transporter protein is found. Replacement of 1,25(OH)$_2$D$_3$ restores taurine uptake by vesicles.

The taurine transporter is regulated by 1,25(OH)$_2$D$_3$ and retinoic acid, and in LLC-PK1 proximal tubule cells *TauT* is upregulated. This regulation appears to require activation of both vitamin D receptors (VDR) and retinoic acid receptors (RXR) and formation of VDR/RXR complexes. It also appears that regulation of the *TauT* gene by 1,25(OH)$_2$D$_3$ and retinoic acid occurs at the transcriptional level via binding of the VDR/RXR complex to AP1 sites on the *TauT* promoter. With adequate 1,25(OH)$_2$D$_3$, taurine transporter protein is found in the apical membrane of the proximal tubule brush border and renal taurine reabsorption is normalized. The studies reported in this review are consistent with that hypothesis.

Acknowledgments The authors wish to thank Andrea B. Patters for editorial assistance.

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The Effect of Perinatal Taurine on Adult Renal Function Does Not Appear to Be Mediated by Taurine's Inhibition of the Renin-Angiotensin System

Sanya Roysommuti, Angkana Kritsongsakchai, and J. Michael Wyss

Abbreviations

ACE	Angiotensin converting enzyme
ACEI	Angiotensin converting enzyme inhibitor
FD	Fetal or prenatal ACEI treatment
i.p.	Intraperitoneal
LD	Lactation or postnatal ACEI treatment
RAS	Renin-angiotensin system
SD	Sprague-Dawley

1 Introduction

In prenatal and early postnatal life, exposure to nutritional and hormonal elements can have long-term consequences and can affect adult function and disease (Baum 2010; Cabral et al.2012; Hogg et al.2012; Roysommuti and Wyss 2014). Like most other organs, renal function is programmed during the perinatal period by several factors (Dotsch et al.2009; Kett and Denton 2011; Woods and Rasch 1998), including dietary taurine exposure and the renin-angiotensin system (RAS), both playing a vital role in renal growth and development (Roysommuti and Wyss 2014).

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Taurine (2-aminoethanesulfonic acid) is a free beta-amino acid found abundantly in all mammalian tissues and is reported to play a vital role from prenatal to adult life (Roysommuti and Wyss 2014). Perinatal taurine deficiency induces low birth weights and these animals have a high risk of adult diseases including coronary vascular diseases, hypertension, diabetes mellitus, and renal dysfunction. In contrast, perinatal taurine supplementation has been shown to promote growth and development. Further, taurine supplementation or diets high in taurine improve congestive heart failure by inhibition of the cardiac RAS and probably also by inhibition of the systemic RAS (Ito et al.2014; Xu et al.2008). Moreover, both taurine supplementation and angiotensin converting enzyme (ACE) inhibition prevent renal damage and dysregulation with advancing age to a similar degree (Cruz et al.2000).

All components of the systemic and local RAS are present and effectively function during perinatal life, and perinatal inhibition of the RAS by either an angiotensin converting enzyme inhibitor (ACEI) or an angiotensin II receptor antagonist can induce renal damage and dysfunction in adult normotensive animals (Guron and Friberg 2000) and humans (Guron et al.2006; Tabacova et al.2003). Perinatal RAS inhibition can result in decreased nephron number, glomerular and tubular damage, reduction of glomerular filtration and renal blood flow (Woods and Rasch 1998). In addition, such perinatal RAS inhibition induces salt-sensitive hypertension in normotensive rat strains (Fang et al.1999). In contrast, lifetime inhibition of RAS improves renal function and protects against damage in spontaneously hypertensive rats (SHR) (Roysommuti et al.1999).

Our previous experiments indicate that perinatal taurine depletion or supplementation alters adult renal function, an effect that is amplified by high sugar intake after weaning (Roysommuti et al.2009, 2010a, b). In female rats that are perinatally depleted of taurine, a high sugar diet blunts baroreflex control of arterial pressure and increases sympathetic nerve activity. All of these are eliminated by acute inhibition of RAS, but not by estrogen receptor blockade (Thaeomor et al.2010). In contrast, perinatal taurine supplementation followed by high sugar intake significantly depresses baroreflex sensitivity without any effect on autonomic nerve activity, and this baroreflex dysfunction is normalized by estrogen receptor blockade but not ACE inhibition (Thaeomor et al.2013). In addition, our previous data indicate that prenatal (versus postnatal) taurine supplementation affects adult renal excretory function differentially (Roysommuti et al.2010a, b). The present study further tests the hypothesis that in male rats, the effect of perinatal taurine supplementation on adult renal function parallels, and probably is primarily mediated by, perinatal inhibition of RAS by taurine.

2 Methods

2.1 Animals

Sprague-Dawley (SD) rats from the animal unit of Faculty of Medicine, Khon Kaen University were bred and maintained at constant humidity (60 ± 5 %), temperature (24 ± 1 °C), and light cycle (0600–1800 h). Female Sprague-Dawley rats were fed

normal rat chow and given water alone (Control) or water containing captopril (an ACEI, 400 mg/ml) from conception until delivery (Fetal RAS depletion, FD) or from delivery until weaning (Lactation RAS depletion, LD). After weaning, the male offspring were fed normal rat chow and tap water *ad libitum*. All experimental procedures were approved by the Khon Kaen University Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health guidelines.

2.2 Experimental Protocol

At 7–8 weeks of age, under sodium pentobarbital anesthesia (Nembutal, 50 mg/kg, i.p.), all male rats were implanted with femoral arterial, venous, and bladder catheters. Forty-eight hours later, each rat was placed in a rat restrainer in which they could move back and forth, and the rats were acclimated to restraint for 1 week (3 h per day) prior to the experiment. Femoral arterial and venous catheters were flushed with 0.9 % heparinized saline (20 units of heparin/ml, 0.2–0.3 ml) to remove blood clotting. The femoral arterial catheter was connected to a pressure transducer (BIOPAC Systems, Goleta, CA, USA) for measurement of arterial pressure and heart rate throughout the experiment and the femoral venous catheter was connected to an infusion pump (Harvard Apparatus, model 975, Boston, Mass, USA). The bladder catheter was then flushed with 0.9 % NaCl, 0.2–0.3 ml. After 5–10 min of rest, an isotonic saline solution containing 0.5 % inulin and 0.5 % *p*-aminohippuric acid (PAH) was intravenously infused at a rate of 0.5 ml/min for 1 min as a priming dose, followed by a basal rate of 20 μl/min for 45 min. A urine sample was collected from the bladder catheter for the last 30 min period, and a blood sample (0.2 ml) was taken at the midpoint for baseline renal function, and the blood lost was replaced with an equal volume of normal saline (via the arterial catheter). After baseline data collection, the isotonic saline solution was intravenously infused at a rate of 0.5 ml/min to the final volume of 5 % body weight, followed by a basal rate of 20 μl/min until the end of the experiment. Urine samples were collected at 15, 30, 60, and 90 min intervals after the initiation of the saline infusion, blood samples were collected (0.2 ml each) at the midpoint of each urine collection interval and replaced with equal volumes of saline. At the end of experiments, all animals were sacrificed with an overdose of sodium pentobarbital and kidney weight (KW) was measured.

2.3 Data Analyses

Mean arterial pressure and heart rate were analyzed by Acknowledge software (BIOPAC Systems). Urine volumes were measured gravitationally, urine and plasma sodium and potassium were assessed by flame photometry, and urine and plasma inulin and PAH by colorimetry. Glomerular filtration rate (GFR) was estimated by inulin clearance, effective renal blood flow (ERBF) by PAH clearance and

hematocrit, effective renal vascular resistance (ERVR) by MAP/ERBF, filtration fraction by GFR/effective renal plasma flow or PAH clearance (%), fractional water excretion by a urine flow to GFR ratio (FE_{H2O}, %), fractional sodium excretion by the ratio of urine sodium excretion to filtered sodium load (FE_{Na}, %), and fractional potassium excretion by the ratio of urine potassium excretion to filtered potassium load (FE_K, %).

All data are expressed as means ± SEM and were statistically analyzed using one-way ANOVA and appropriate *post hoc* Duncan's Multiple Range test with a significant criterion of p-value less than 0.05 (Statmost version 3.5, Dataxiom Software, USA).

3 Results

Compared to control groups, perinatal inhibition of the RAS by captopril significantly decreased body weight (Control, 200 ± 11 g; FD, 164 ± 6 g; LD, 165 ± 5 g; $P < 0.05$) but significantly increased kidney to body weight ratios (Control, 0.32 ± 0.01 g; FD, 0.40 ± 0.02 g; LD, 0.41 ± 0.03 g; $P < 0.05$). Neither prenatal nor postnatal captopril treatment significantly affected mean arterial pressure, heart rate (Fig. 1), effective renal blood flow, or effective renal vascular resistance (Fig. 2) in adult rats at rest or after isotonic saline load. Compared to Control, glomerular filtration rate significantly increased at rest and after saline load in LD and increased only after saline load in FD groups (Fig. 3). However, neither FD nor LD affected filtration fraction throughout the study.

Water excretion significantly increased at rest and 15 min after saline infusion only in FD compared to Control group, while fractional water excretion was significantly increased at rest in FD and at 30 min after a saline load in both FD and LD groups (Fig. 4). Further, sodium excretion significantly increased only after a saline load in FD compared to Control and LD groups, while both FD and LD compared to Control displayed significant decreases in fractional sodium excretion 30 min after saline load (Fig. 5). In contrast to sodium and water excretion, potassium excretion significantly increased both at rest and after saline load in both FD and LD compared to Control groups (Fig. 6). Perinatal inhibition of the RAS significantly increased fractional potassium excretion at rest, but not after saline load in FD and significantly decreased fractional potassium excretion in LD 90 min after saline load but not at rest.

We next compared whether the effects of perinatal RAS inhibition on adult renal function were parallel to those of perinatal taurine supplementation previously reported in our parallel studies (Roysommuti et al.2010a, b). Table 1 summarizes the effect of pre- and postnatal RAS inhibition or taurine supplementation on adult renal function. The data indicate that the two treatments had differing effects in 54 % of the indices measured. Prenatal treatments with captopril versus taurine differentially affected 67 % of the indices, while postnatal treatment differentially affected 42 % of indices. In no case did the two treatments cause a similar change in renal function.

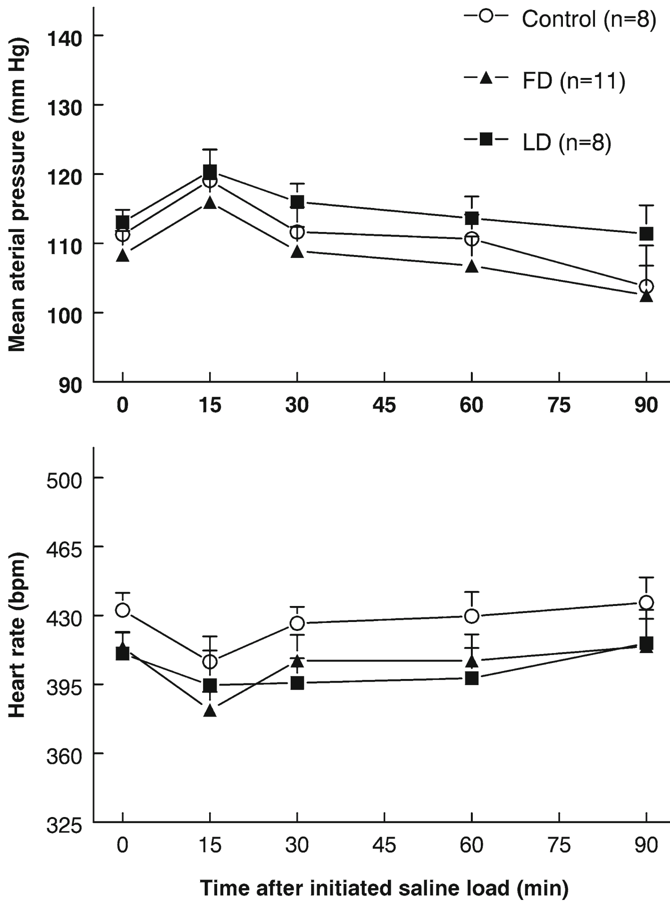

Fig. 1 Mean arterial pressure (*upper*) and heart rate (*lower*) at rest and after acute saline infusion in Control, prenatal (Fetus, FD), and postnatal (Lactation, LD) renin-angiotensin system inhibition groups. No significant difference was observed among the three groups

4 Discussion

Taurine and RAS inhibition have many similar effects on the regulation of cardiovascular and renal function, and they have both been reported to affect growth and development of the kidney (Kett and Denton 2011; Roysommuti and Wyss 2014). In adult rats, either taurine exposure or RAS inhibition prevents renal dysregulation with age (Cruz et al.2000). During perinatal life, various influences program renal excretory and hormonal function, and inappropriate regulation can lead to renal disorders in adult life (Roysommuti and Wyss 2014).

In adults, taurine supplementation can inhibit adverse RAS effects in several disorders including cardiac hypertrophy and heart failure (Ito et al.2014), and previous

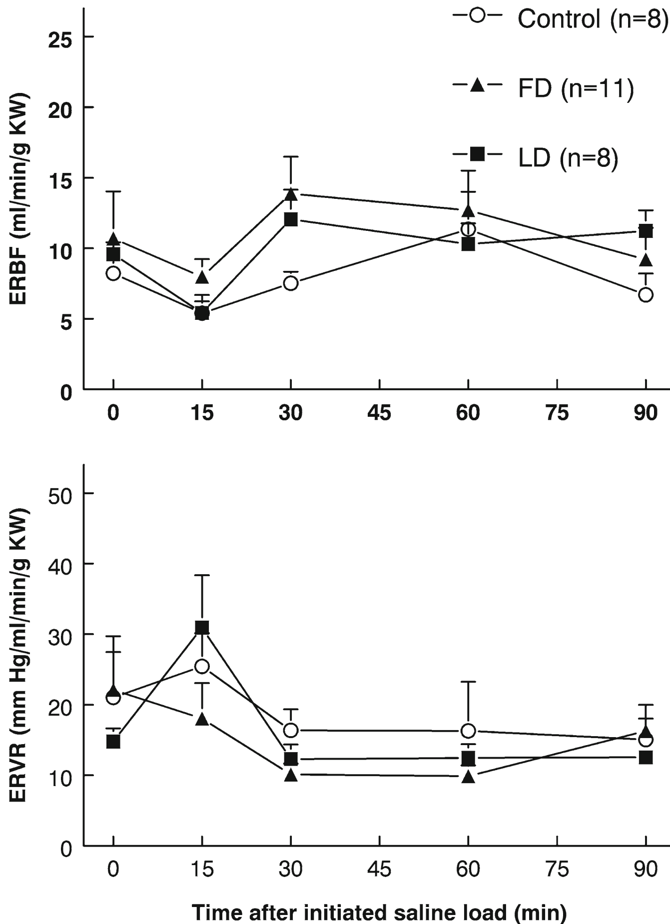

Fig. 2 Effective renal blood flow (ERBF; *upper*) and effective renal vascular resistance (ERVR; *lower*) at rest and after acute saline infusion in Control, prenatal (Fetus, FD), and postnatal (Lactation, LD) renin-angiotensin system inhibition groups. No significant difference was observed among the three groups

experiments indicate that in female rats, perinatal taurine depletion followed by high sugar intake after weaning alters adult arterial pressure control via RAS overactivity (Thaeomor et al.2010). Although the present study indicates that both perinatal taurine and RAS alter adult renal function, the majority of the changes in these parameters are different between the treatments. In the present study, not a single parameter measured was negatively or positively affected by both treatments. Thus, the data are not consistent with the hypothesis that perinatal taurine supplementation affects adult renal function primarily via perinatal inhibition of the RAS, at least in male rats. Further, this study also suggests that the RAS effects on renal function are more frequent than those following taurine supplementation.

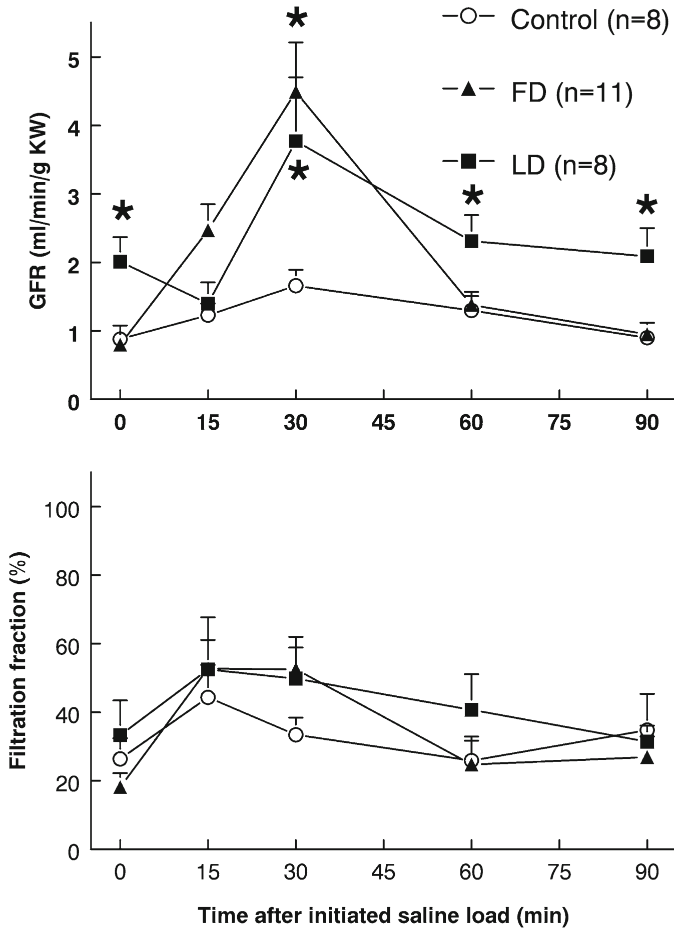

Fig. 3 Glomerular filtration rate (GFR; *upper*) and filtration fraction (*lower*) at rest and after acute saline infusion in Control, prenatal (Fetus, FD), and postnatal (Lactation, LD) renin-angiotensin system inhibition groups (*$P<0.05$ compared to Control)

In humans, nephrogenesis starts around 5 weeks of gestation and the main structure of the kidney is completed before birth (Kett and Denton 2011). This growth and development includes forming of blood vessels, glomeruli, tubules, hormonal networks, and neural innervation. In most animals and particularly in rodents, the nephrogenesis starts prenatally and is complete before weaning. However, maturation of nephrons continues several months after birth in humans, and in rodents is complete shortly after weaning. Thus, either prenatal or postnatal RAS inhibition or taurine supplementation (Roysommuti et al.2010a, b) has the potential to affect renal function in adult rats. The main perinatal effect of RAS inhibition in the present study is on alteration of glomerular and tubular function, supporting previous experiments showing that RAS affects nephrogenesis and maturation (Baum 2010;

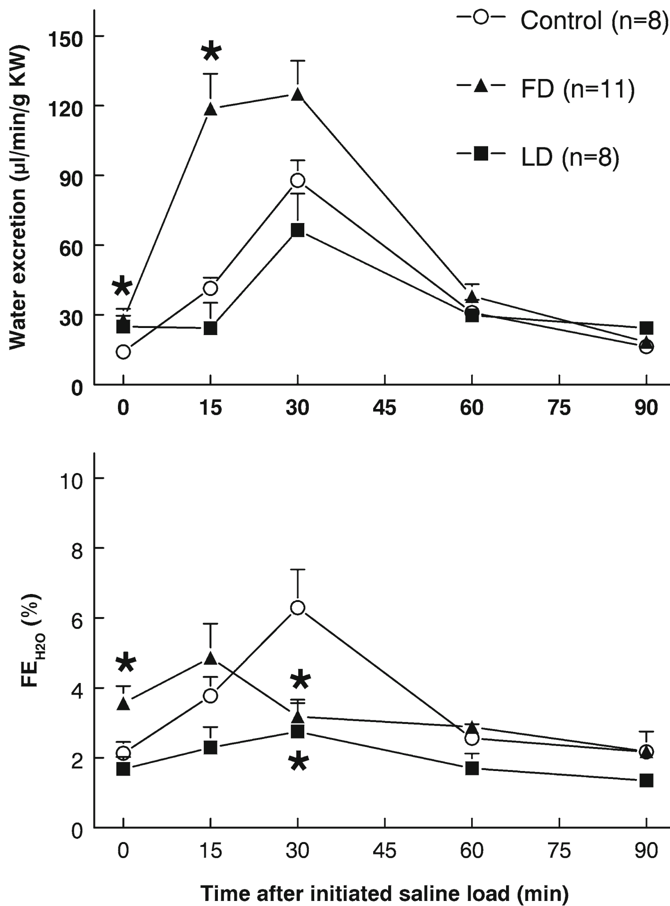

Fig. 4 Water excretion (*upper*) and fractional water excretion (FE_{H2O}; *lower*) at rest and after acute saline infusion in Control, prenatal (Fetus, FD), and postnatal (Lactation, LD) renin-angiotensin system inhibition groups (* $P<0.05$ compared to Control)

Gubler and Antignac 2010; Guron and Friberg 2000; Spaggiari et al.2012; Woods and Rasch 1998).

Perinatal inhibition of RAS induces decreased nephron number, increased glomerular hyperfiltration, and increased tubular sodium reabsorption in adult life (Kett and Denton 2011). These changes may explain why GFR increased and fractional sodium and water excretion decreased after an isotonic saline challenge in captopril-treated rats. Under normal conditions, acute isotonic volume expansion decreases angiotensin II and renal sympathetic nerve activity, leading to decreased renal vascular resistance, increased renal blood flow, and increased glomerular filtration, respectively (see control group data). These neurohormonal responses also result in decreased tubular water and sodium reabsorption, thus producing diuresis and natriuresis. The perinatal RAS effects suggest that marked increases in GFR

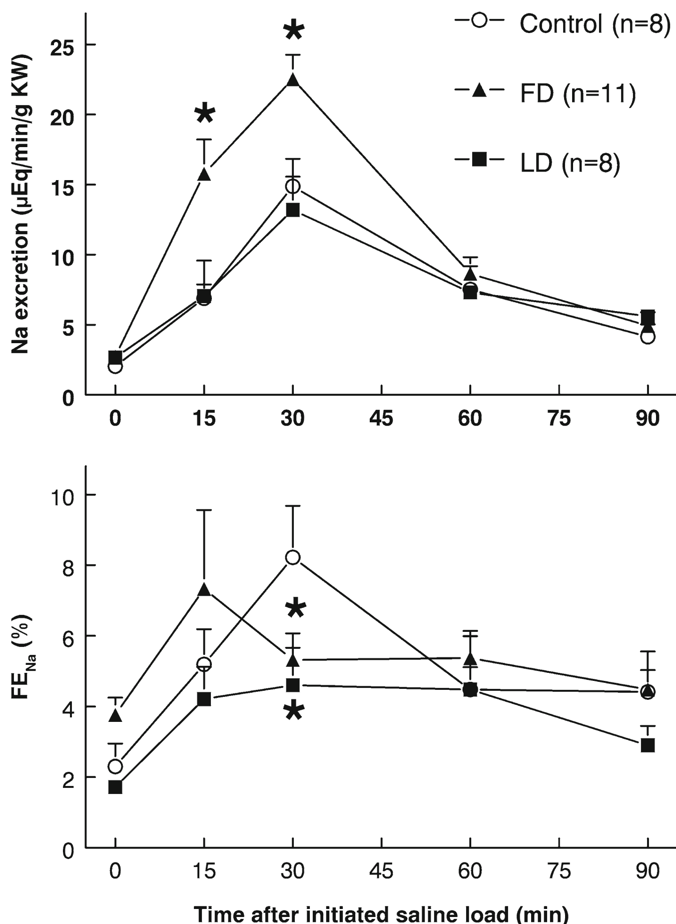

Fig. 5 Sodium excretion (*upper*) and fractional sodium excretion (FE$_{Na}$; *lower*) at rest and after acute saline infusion in Control, prenatal (Fetus, FD), and postnatal (Lactation, LD) renin-angiotensin system inhibition groups (*$P<0.05$ compared to Control)

and tubular water and electrolyte reabsorption may be due to an inappropriate RAS response to volume expansion in adult rats.

RAS underlies cardiac hypertrophy and heart failure in several cardiovascular disorders, and thus ACE inhibition or angiotensin II receptor antagonists are generally prescribed to treat these patients (Escobar and Barrios 2013; Ferrari and Boersma 2013; Segura et al.2013). Several lines of evidence indicate that in adults, taurine supplementation also improves cardiac ischemia/reperfusion and heart failure by inhibiting RAS activation (Ito et al.2014). In animal models, chronic taurine supplementation decreases cardiac ischemia/reperfusion injury (Sahin et al.2011), and acute treatment before (versus after) an episode of cardiac ischemia/reperfusion is less effective (Ueno et al.2007). However, taurine may more directly inhibit oxidative

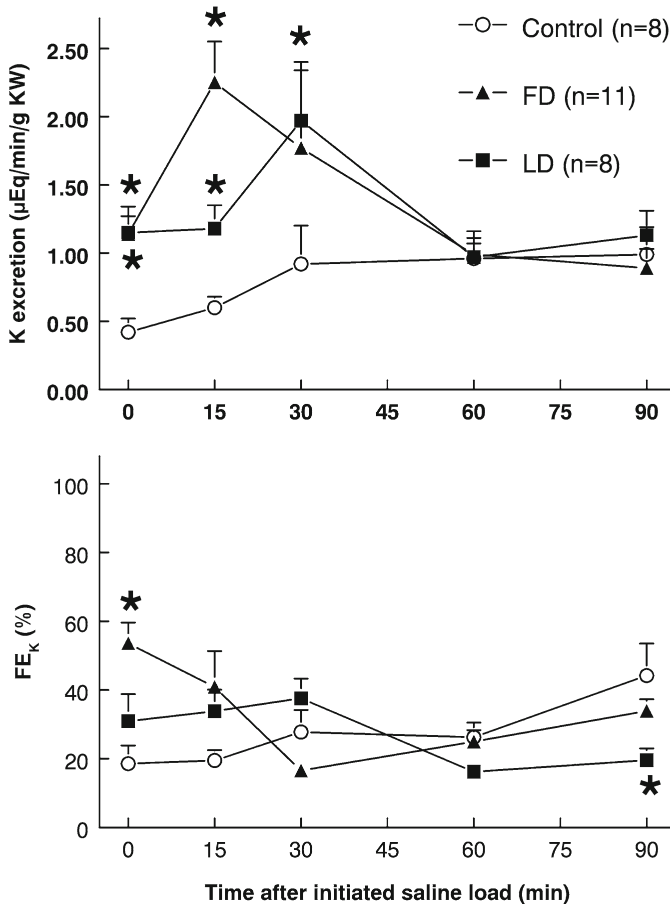

Fig. 6 Potassium excretion (*upper*) and fractional potassium excretion (FE_K; *lower*) at rest and after acute saline infusion in Control, prenatal (Fetus, FD), and postnatal (Lactation, LD) renin-angiotensin system inhibition groups (*$P<0.05$ compared to Control)

stress either through, or outside of, its RAS inhibitory action (Ueno et al.2007). Taurine is an antioxidant (Roysommuti and Wyss 2014), whereas angiotensin II increases oxidative stress (Kopkan and Cervenka 2009; Sachse and Wolf 2007). In adult kidneys, angiotensin II increases water and sodium reabsorption directly or indirectly via stimulation of aldosterone, whereas taurine supplementation decreases sodium and probably water reabsorption (Roysommuti and Wyss 2014).

These lines of evidence led us to the hypothesis that perinatal taurine supplementation could inhibit the RAS in the adult and might thereby have beneficial long-term effects. Thus, perinatal taurine supplementation might provide an alternative mechanism for RAS inhibition in the adult, particularly in heart disease (Ito et al.2014). In contrast to this hypothesis, the present data indicate that taurine sup-

Table 1 Summary of changes in renal function after prenatal (Fetus, FD) and postnatal (Lactation, LD) caused by renin-angiotensin system inhibition (Captopril) and taurine supplementation (Taurine)

	Prenatal treatment				Postnatal treatment			
	Resting		Post-infusion		Resting		Post-infusion	
Parameter	Captopril	Taurine	Captopril	Taurine	Captopril	Taurine	Captopril	Taurine
MAP	N	I	N	I	N	I	N	I
HR	N	N	N	N	N	N	N	N
ERBF	N	D	N	D	N	N	N	N
ERVR	N	I	N	N	N	N	N	I
GFR	N	N	I	N	I	N	I	N
FF	N	I	N	N	N	N	N	N
E_{H2O}	I	N	I	N	N	N	N	N
FE_{H2O}	I	N	D	I	N	N	D	N
E_{Na}	N	N	I	N	N	N	N	N
FE_{Na}	N	N	D	N	N	N	D	N
E_K	I	N	I	N	I	N	I	N
FE_K	I	N	N	N	N	N	D	N

All changes noted refer to significant changes from Control (untreated) rats
MAP mean arterial pressure, *HR* heart rate, *ERBF* effective renal blood flow, *ERVR* effective renal vascular resistance, *GFR* glomerular filtration rate, *FF* filtration fraction, E_{H2O} water excretion, FE_{H2O} fractional water excretion, E_{Na} sodium excretion, FE_{Na} fractional sodium excretion, E_K potassium excretion, FE_K fractional potassium excretion, *N* normal, *D* decrease, *I* increase (with reference to Control group)

plementation and ACE inhibition during pre- or postnatal period affect adult renal function quite differently. In addition, the most deleterious effects of either treatment on adult renal function are observed after prenatal rather than postnatal inhibition. Thus, the RAS and taurine are critical during perinatal life and alterations in their availability can predispose to adult renal dysfunction.

Our previous experiments indicate that either prenatal or postnatal taurine supplementation slightly and significantly increases mean arterial pressure but not heart rate in adult, male rats (Roysommuti et al.2010a). Further, perinatal taurine supplementation slightly blunts baroreflex control of the renal nerve in adult female rats, and this effect is abolished by an estrogen receptor blocker tamoxifen, but not captopril (Thaeomor et al.2013). In contrast to taurine, perinatal inhibition of the RAS did not affect adult arterial pressure and heart rate, an effect that is similar to previous reports (Fang et al.1999). In addition, the offspring who received perinatal RAS inhibition displayed increased salt-sensitive hypertension following 2-weeks of drinking isotonic saline (unpublished data) or eating high salt diets (Fang et al.1999). Although perinatal taurine supplementation does not induce salt-sensitive hypertension, taurine supplementation in late pregnant rats stimulates postnatal growth and induces obesity and insulin resistance in adult offspring (Hultman et al.2007). Moreover, in adult SHR, taurine supplementation accelerates the hypertensive response to a high salt diet during nighttime but not daytime (Suwanich et al.2013).

5 Conclusion

Both taurine and the renin-angiotensin system possess several important functions in humans and animals, especially during perinatal development. For instance, both perinatal taurine supplementation and RAS inhibition prevent age-related renal dysfunction and renal dysfunction in hypertensive, glucose intolerant rats. Further, perinatal taurine supplementation blunts cardiac injury and cardiac ischemia/reperfusion dysfunction and heart failure by inhibition of RAS. However, both perinatal RAS inhibition and taurine supplementation can also lead to adult renal dysfunction. The present study indicates that while perinatal taurine supplementation can alter renal function in adults, it likely does so through mechanisms other than RAS inhibition.

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Perinatal Taurine Depletion Alters the Renal Excretory Effect of the Renin-Angiotensin System in Adult Female Rats

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Abbreviations

$AT_{1,1b,2}$ receptor	Angiotensin II receptor subtype 1, 1b or 2, respectively
ACEI	Angiotensin converting enzyme inhibitor
C	Control
Cap	Captopril
CG	Control plus high sugar intake
CW	Control without high sugar intake
ERBF	Effective renal blood flow
ERVR	Effective renal vascular resistance
$FE_{H2O, K, Na}$	Fractional water, potassium or sodium excretion, respectively
GFR	Glomerular filtration rate
HW	Heart weight
i.p.	Intraperitoneal
IUGR	Intrauterine growth restriction
KW	Kidney weight
MAP	Mean arterial pressure

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PAH	*p*-Aminohippuric acid
RAS	Renin-angiotensin system
TD	Perinatal taurine depletion
TDG	TD plus high sugar intake
TDW	TD without high sugar intake

1 Introduction

In humans, nephrogenesis starts around 5 weeks of gestation and the main renal structure is completed before birth (Dotsch et al. 2009; Kett and Denton 2011). Renal growth and development include formation of blood vessels, glomeruli, tubules, hormonal networks, and innervation. In most animals particularly rodents, nephrogenesis starts prenatally and is complete before weaning; however, maturation of nephrons continues until shortly after weaning in rodents but lasts for several months after birth in humans. Thus, perinatal nutritional and hormonal imbalances would be expected to affect adult renal function and health (Roysommuti and Wyss 2014).

A clue to the role of perinatal dietary taurine and the angiotensin system in the process of renal development is suggested by studies of intrauterine growth restriction (IUGR), which decreases taurine and induces AT_{1b} receptor mRNA and protein overexpression in the adrenal gland of adult offspring, likely by epigenetic mechanisms (Bogdarina et al. 2007). In contrast, IUGR reduces renin mRNA, AT_1 receptor protein in the adult kidney and increases angiotensin II levels and AT_2 receptor mRNA (Vehaskari et al. 2004; Woods et al. 2001). Further, IUGR decreases intrarenal renin and angiotensin II content in male but not female offspring (Woods et al. 2005). This suggests that gender affects renal renin-angiotensin system (RAS) programming.

In adults following a perinatal renal insult, the kidney and related control mechanisms tend to compensate, in part depending on exposure to several environmental factors, e.g., nutritional factors, neuroendocrine status, and individual behavior (Baum 2010; Kett and Denton 2011). For example, perinatal RAS inhibition produces offspring that are sensitive to development of hypertension when placed on a high (but not basal) salt diet (Fang et al. 1999). Lifetime inhibition of the RAS from conception onward prevents renal dysfunction and damage in adult SHR (Roysommuti et al. 1999), but fails to prevent hypertensive responses to a high salt diet or the adverse consequences thereof (Wyss et al. 1994).

Epidemiological studies suggest a negative relationship between the incidence of cardiovascular diseases and consumption of diets high in taurine, particularly fish (Yamori et al. 2010). Adult consumption of high taurine diets decreases the rate of organ damage that normally accompanies advancing age. Especially protected in this process are brain, kidneys, and heart (Roysommuti and Wyss 2014). Further, hypertension in animal models can also be prevented or reduced by a taurine supplemented diet (Militante and Lombardini 2002), and our previous experiments

indicate that perinatal taurine deficiency impairs renal function in adult male and female rats (Roysommuti et al. 2009a, 2010a) and impairs the autonomic nervous system in adult male (Roysommuti et al. 2009b) but not female rats (Thaeomor et al. 2010). Also, in both male and female rats, sympathetic nerve activity becomes hyperactive following treatment with a high sugar diet from weaning onward. Baroreflex sensitivity controls of heart rate and/or renal nerve activity are also blunted in these rats (Roysommuti et al. 2009b; Thaeomor et al. 2010). In adult female rats, these baroreflex abnormalities can be normalized by acute captopril, but not by tamoxifen, treatment (Thaeomor et al. 2010), suggesting that RAS overactivity underlies these baroreflex abnormalities. Whether this RAS overactivity also underlies renal impairment following perinatal taurine depletion has not been tested.

Although captopril treatment decreases mean arterial pressure in control rats, control rats receiving high sugar intake after weaning, and perinatal taurine depleted rats, the treatment does not affect mean arterial pressure in the perinatal taurine depleted rats on high sugar intake (Thaeomor et al. 2010). These data suggest that the effect of RAS overactivity on the autonomic nervous system and baroreflex sensitivity *per se* is not sufficient to alter arterial pressure in these rats. In addition, high sugar intake, similar to that used in the present study, impairs renal function without affecting arterial pressure and glucose tolerance and does this via RAS overactivity in adult, male rats (Roysommuti et al. 2002). These different effects indicate that systemic compared to local RAS functions are differentially regulated (Ferrario et al. 2014). For example, high salt intake during pregnancy induces high numbers of angiotensin II-positive cortical cells but lowers circulating angiotensin I in adult male Wistar rats (Cabral et al. 2012).

The present study tests the hypothesis that perinatal taurine depletion impairs renal excretory function by increasing RAS activity in adult female rats and that this is exacerbated by a high sugar diet.

2 Methods

2.1 Animals

Sprague-Dawley rats were bred from the Northeast Laboratory Animal Center, Khon Kaen University and maintained at constant humidity (60 ± 5 %), temperature (24 ± 1 °C), and light cycle (0600–1800 h). Female and male Sprague-Dawley rats, weighing 200–250 g were randomly assigned into a mating procedure. After conception, each female rat was separated, caged individually, and fed normal rat chow and given water alone (C) or water containing 3 % beta-alanine (taurine depletion, TD) from conception until weaning. After weaning, the rats received normal rat chow and tap water with (CG, TDG) or without 5 % glucose (CW, TDW) throughout the study. Beginning a week before renal study, half of the rats in each treatment were continuously treated with an angiotensin converting enzyme inhibitor (ACEI) in tap water (captopril, 400 mg/l) throughout the experiment (CW + Cap, CG + Cap,

TDW + Cap, TDG + Cap). All experimental procedures were approved by the Khon Kaen University Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health guidelines.

2.2 Experimental Protocol

At 7–8 weeks of age, under sodium pentobarbital anesthesia (Nembutal, 50 mg/kg, i.p.), all female rats were implanted with femoral arterial, venous, and bladder catheters (Roysommuti et al. 2002). Forty-eight hours later, each rat was placed in a rat restrainer in which they could move back and forth, a condition to which all rats had been acclimated 3 h per day for 1 week prior to the renal function study. After flushing the catheters, the femoral arterial catheter was connected to a pressure transducer (BIOPAC Systems, Goleta, CA, USA) for continuous measurement of arterial pressure and heart rate throughout the experiment and the femoral venous catheter was connected to an infusion pump (Harvard Apparatus, model 975, Boston, Mass, USA). After 5–10 min of rest, an isotonic saline solution containing 0.5 % inulin and 0.5 % *p*-aminohippuric acid (PAH) was intravenously infused at a rate of 0.5 ml/min for 1 min as a priming dose, followed by a basal rate of 20 μl/min for 45 min. A urine sample was collected from the bladder catheter for the last 30 min period, and an arterial blood sample (0.2 ml) was taken at the midpoint for baseline renal function, and the blood lost was replaced with an equal volume of normal saline. After baseline data collection, the isotonic saline solution was intravenously infused at a rate of 0.5 ml/min to the final volume of 5 % body weight, followed by a basal rate 20 μl/min until end of the experiment. Urine samples were collected at 15, 30, 60, and 90 min intervals after the initiation of the saline infusion, arterial blood samples were collected (0.2 ml each) at the midpoint of each urine collection interval and replaced with equal volumes of saline. At the end of the experiments, all animals were sacrificed with an overdose of sodium pentobarbital and kidney (KW) and heart weights (HW) were measured.

2.3 Data Analyses

Mean arterial pressure and heart rate were analyzed by Acknowledge software (BIOPAC Systems). Urine volumes were measured gravitationally, urine and plasma sodium and potassium were assessed by the Srinagarind Hospital Laboratory Unit (Faculty of Medicine, Khon Kaen University), and urine and plasma inulin and PAH by colorimetry. Glomerular filtration rate (GFR) was estimated by inulin clearance, effective renal blood flow (ERBF) by PAH clearance and hematocrit, effective renal vascular resistance (ERVR) by MAP/ERBF, filtration fraction by GFR/effective renal plasma flow or PAH clearance, fractional water excretion by a urine flow to GFR ratio (FE_{H2O}, %), fractional sodium excretion by the ratio of urine sodium excretion to filtered sodium load (FE_{Na}, %), and fractional potassium excretion by the ratio of urine potassium excretion to filtered potassium load (FE_{K}, %).

All data are expressed as means ± SEM and were statistically analyzed using one-way ANOVA and appropriate *post hoc* Tukey's test with a significant criterion of p-value less than 0.05 (Statmost version 3.5, Dataxiom Software, USA).

3 Results

At 7–8 weeks of age, body weight (CW, 194 ± 3 g; CG, 186 ± 2 g; TDW, 197 ± 3 g; TDG, 185 ± 2 g; CW + Cap, 194 ± 3 g; CG + Cap, 193 ± 5 g; TDW + Cap, 195 ± 5 g; TDG, 192 ± 3 g), kidney weight (CW, 1.60 ± 0.02 g; CG, 1.60 ± 0.04 g; TDW, 1.57 ± 0.02 g; TDG, 1.54 ± 0.02 g; CW + Cap, 1.61 ± 0.03 g; CG + Cap, 1.57 ± 0.04 g; TDW + Cap, 1.59 ± 0.05 g; TDG + Cap, 1.62 ± 0.02 g), and heart weights (data not shown) were not significantly different among the eight groups. Mean arterial pressures at rest and after a saline load were not significantly different among CW, CG, TDW, and TDG groups, but after captopril treatment, mean arterial pressures were decreased in all groups compared to their associated captopril untreated groups (Fig. 1). TDW + Cap compared to the other groups showed a much greater reduction in arterial pressure 30 min after the saline load (about 12 mm Hg below others;

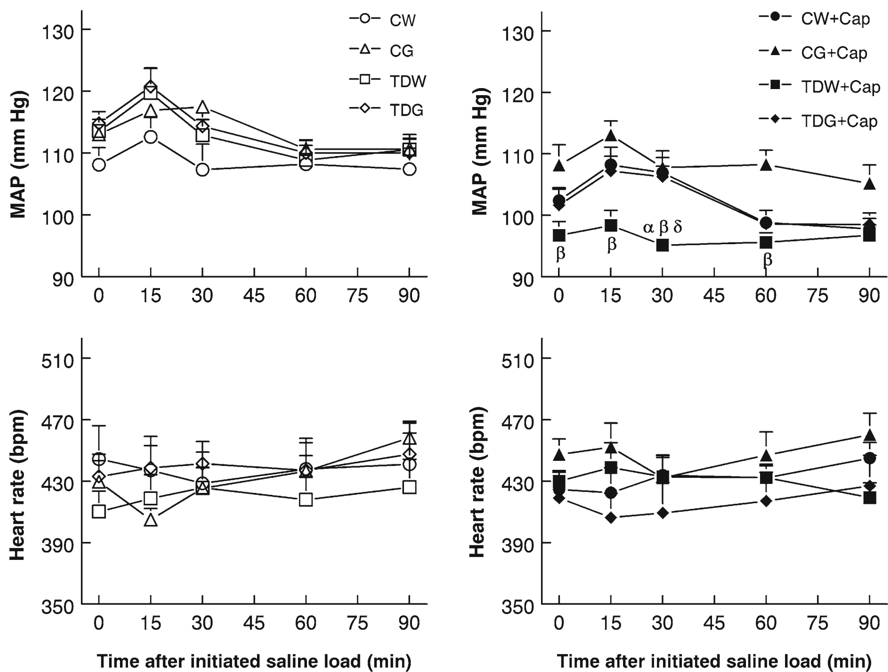

Fig. 1 Mean arterial pressure (*upper two*) and heart rate (*lower two*) at rest and in response to an acute saline load in control (CW), control plus high sugar intake (CG), perinatal taurine depletion (TDW), and perinatal taurine depletion plus high sugar intake (TDG) with (*right two*; +Cap) or without (*left two*) captopril treatment (an angiotensin converting enzyme inhibitor); α·β·δ$P < 0.05$ compared to CW or CW + Cap, CG or CG + Cap, and TDW or TDW + Cap, respectively

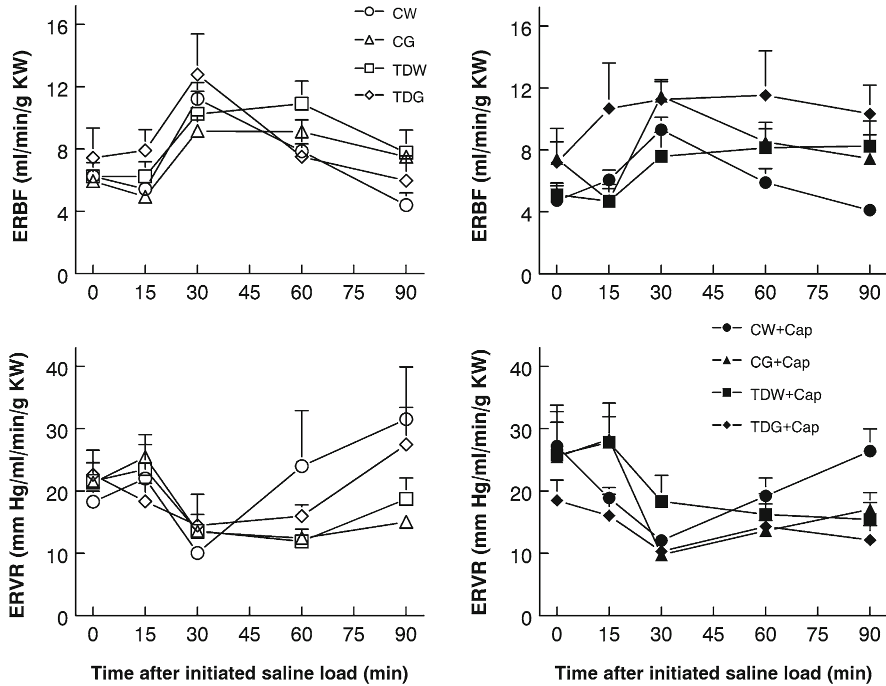

Fig. 2 Effective renal blood flow (ERBF; *upper two*) and effective renal vascular resistance (ERVR; *lower two*) at rest and in response to an acute saline load in control (CW), control plus high sugar intake (CG), perinatal taurine depletion (TDW), and perinatal taurine depletion plus high sugar intake (TDG) with (*right two*; +Cap) or without (*left two*) captopril treatment (an angiotensin converting enzyme inhibitor). No significant difference is observed among the eight groups

$p<0.05$, Fig. 1). Heart rates before and after captopril treatments were not significantly different among the groups. Further, ERBF, ERVR (Fig. 2), GFR, filtration fraction (Fig. 3), and water (Fig. 4) and sodium excretion (Fig. 5) were not different among the groups.

Compared to control rats, fractional water excretion after a saline load (but not at rest) was significantly decreased in CG, TDW, and TDG groups. In response to captopril treatment, the control rats' fractional excretion of water decreased to the levels of the other groups, and there were no differences among the four groups of captopril treated rats at rest or during the 60 min post saline infusion (Fig. 4). At 90 min post saline load, fractional water excretion was slightly and significantly higher in the CG+Cap group compared to the CW+Cap, TDW+Cap, and TDG+Cap groups.

Compared to the CW, fractional sodium excretions after a saline load but not at rest were significantly decreased in the CG, TDW, and TDG groups. Further, except for the change in CW vs. CW+Cap rats, there were no captopril induced changes in fractional sodium excretion at any of the time points (Fig. 5).

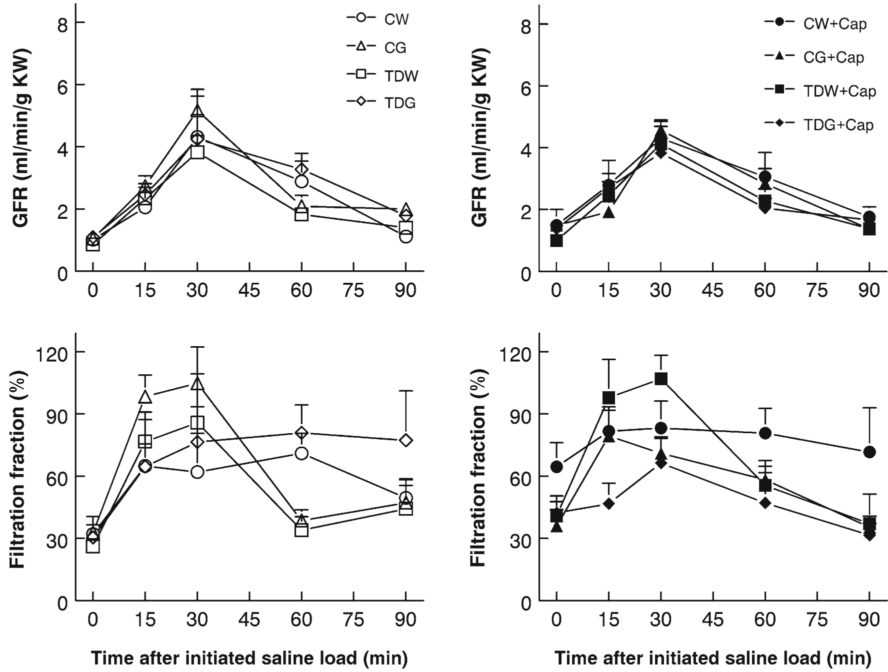

Fig. 3 Glomerular filtration rate (GFR; *upper two*) and filtration fraction (*lower two*) at rest and in response to an acute saline load in control (CW), control plus high sugar intake (CG), perinatal taurine depletion (TDW), and perinatal taurine depletion plus high sugar intake (TDG) with (*right two*; +Cap) or without (*left two*) captopril treatment (an angiotensin converting enzyme inhibitor). No significant difference is observed among the eight groups

In the controls, potassium excretion was not significantly different among the four groups throughout the study, but fractional potassium excretion was significantly different between the CG and TDW groups at 90 min post saline load (Fig.6). The captopril treatment significantly decreased potassium excretion at rest in CG+Cap compared to CW+Cap and at 60 min post saline load, in TDG+Cap compared to TDW+Cap groups, consistent with a change in fractional potassium excretion.

4 Discussion

In the prenatal and early postnatal environment, nutritional and hormonal exposures affect adult renal function and disease (Kett and Denton 2011). Previous studies report that perinatal taurine excess or deficit alters adult renal function in a sex specific manner (Roysommuti et al.2009a, 2010a). In female rats, perinatal taurine

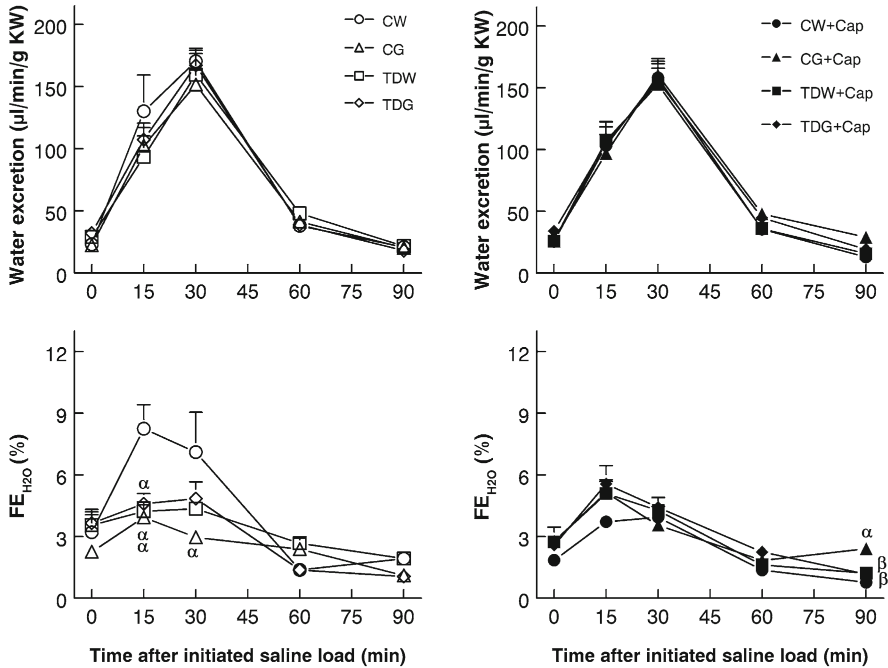

Fig. 4 Water excretion (*upper two*) and fractional water excretion (FE_{H2O}; *lower two*) at rest and in response to an acute saline load in control (CW), control plus high sugar intake (CG), perinatal taurine depletion (TDW), and perinatal taurine depletion plus high sugar intake (TDG) with (*right two*; +Cap) or without (*left two*) captopril treatment (an angiotensin converting enzyme inhibitor); $\alpha{\cdot}\beta P<0.05$ compared to CW or CW + Cap and CG or CG + Cap, respectively

depletion followed by high sugar intake after weaning depresses baroreceptor reflex sensitivity (Thaeomor et al. 2010) and increases sympathetic nerve activity (unpublished data) without any effect on arterial pressure (Thaeomor et al. 2010). Further, inhibition of RAS by an ACEI captopril in these rats normalizes baroreflex function and autonomic nerve activity without any effect on mean arterial pressure, despite being decreased in untreated control rats. The present study indicates that the RAS does not affect renal excretory impairment in adult female rats that were perinatally depleted of taurine, irrespective of high sugar intake. Together, these data suggest that the RAS's ability to disturb the autonomic nervous system and baroreflex function following perinatal taurine depletion and a high sugar diet since weaning is system specific, and thus alters some effects, but not other closely related effects, in adult female rats.

Pressure-regulated diuresis/natriuresis is a primary mechanism that modifies arterial pressure regulation by the kidney (Brands 2012). Thus, blunted pressure-diuresis/natriuresis underlies hypertension in many human and animal models (Hall et al. 2012). In SHR, lifetime inhibition of the RAS by captopril from conception

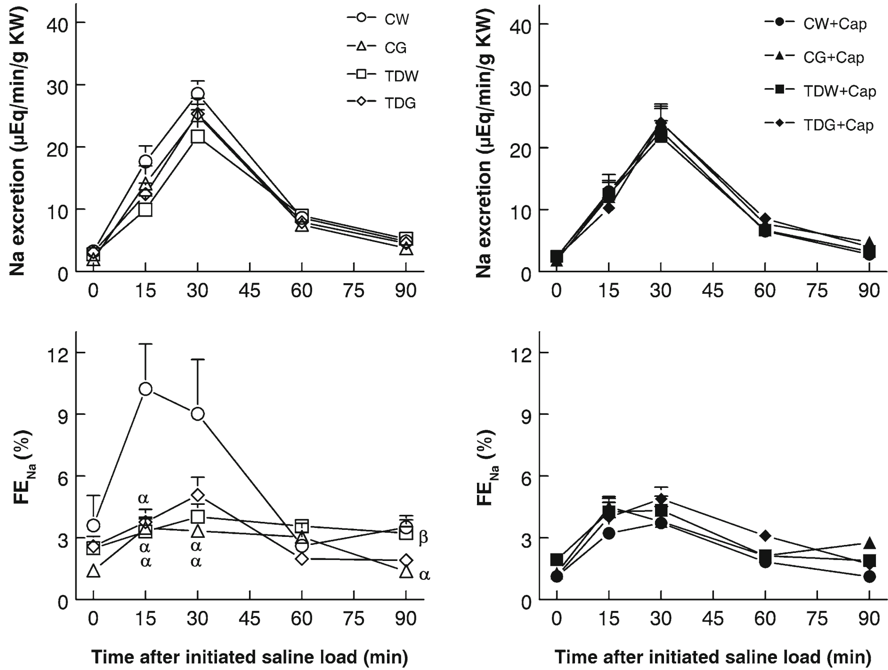

Fig. 5 Sodium excretion (*upper two*) and fractional sodium excretion (FE_{Na}; *lower two*) at rest and in response to an acute saline load in control (CW), control plus high sugar intake (CG), perinatal taurine depletion (TDW), and perinatal taurine depletion plus high sugar intake (TDG) with (*right two*; +Cap) or without (*left two*) captopril treatment (an angiotensin converting enzyme inhibitor); $\alpha{\cdot}\beta P<0.05$ compared to CW or CW+Cap and CG or CG+Cap, respectively

onward normalizes arterial pressure (Wyss et al. 1994), but it does not alter the diuretic and natriuretic responses to an acute saline load (Roysommuti et al. 1999), suggesting ACEI improves pressure-diuresis/natriuresis in these animals (i.e., the renal responses are not decreased by the decreased arterial pressure). However, in SHR, the lifetime captopril treatment leads to salt-sensitive hypertension that is abolished by acute inhibition of sympathetic nervous system activity (Wyss et al. 1995). These data suggest that heightened RAS and sympathetic nerve activity in the SHR are not always linked in their effects on the kidney, but rather have specific effects that are likely related to local actions.

Diuresis and natriuresis following an acute saline load has been shown to depend on several factors particularly atrial natriuretic hormone (Andersen et al. 1998; Cowley et al. 1988), RAS (Roysommuti et al. 1999, 2002; Sandgaard et al. 2005), renal sympathetic nerve activity (Johns et al. 2011), the intrarenal dopamine system, nitric oxide (Costa et al. 2006), prostaglandins (Agnoli et al. 2001), and colloid osmotic pressure (Cowley and Skelton 1991). In dogs, this response is about 40–50 % dependent on increased atrial natriuretic hormone, which decreases tubular water

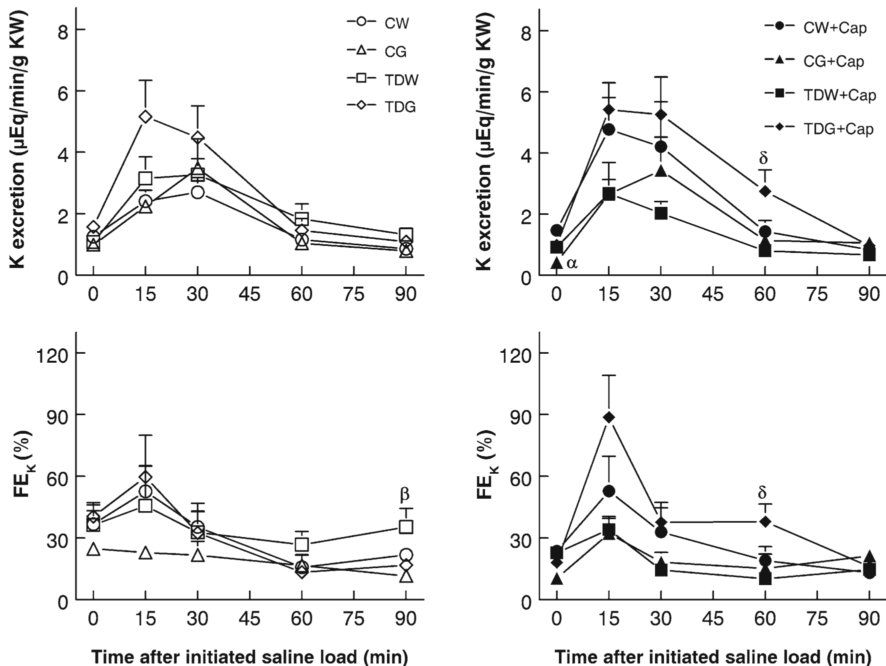

Fig. 6 Potassium excretion (*upper two*) and fractional potassium excretion (FE_K; *lower two*) at rest and in response to an acute saline load in control (CW), control plus high sugar intake (CG), perinatal taurine depletion (TDW), and perinatal taurine depletion plus high sugar intake (TDG) with (*right two*; +Cap) or without (*left two*) captopril treatment (an angiotensin converting enzyme inhibitor); β·δ $P<0.05$ compared to CG or CG+Cap and TDW or TDW+Cap, respectively

and sodium reabsorption (Cowley et al. 1988). Renal blood flow and arterial pressure also are not altered by an acute saline load (Cowley et al. 1988; Sandgaard et al. 2005), as reported in the present study for rats.

In the present data, compared to CW, perinatal taurine depletion treatment decreased fractional water and sodium excretion (indicating increased tubular water and sodium reabsorption), and this parameter was not affected by captopril treatment, except in the CW+Cap group in which inhibition of RAS caused a reduction to the responses. These data suggest that perinatal taurine depletion might depress adult RAS or alter other renal mechanisms in response to an acute saline challenge. Although captopril treatment normalizes autonomic nerve function in TDG rats (Thaeomor et al. 2010), increased renal sympathetic nerve activity following the acute saline load cannot be excluded in this study. The roles of atrial natriuretic peptide, dopamine system, nitric oxide, and prostaglandins on renal function also have to be further clarified.

Potassium excretion is less affected by perinatal taurine depletion, in agreement with previously reported studies (Roysommuti et al. 2010b). However, a significant

increase in potassium excretion consistent with fractional potassium excretion occurs in response to an acute saline load in TDW + Cap compared to TDG + Cap, suggesting that the RAS plays a significant role in potassium excretion, particularly tubular potassium transport, in adult female rats perinatally depleted of taurine. This effect is modified by high sugar intake. Nevertheless, these changes were not significantly different from CW + Cap and CG + Cap rats.

In the *in vitro* experiments, high glucose (25 mM) evokes reactive oxygen species generation and p38 MAPK phosphorylation, as well as stimulates immunoreactive rat angiotensin secretion and angiotensin mRNA expression in renal proximal tubular cells. These effects are blocked by antioxidants (taurine and tiron) (Hsieh et al. 2002). Thus, the intrarenal RAS, rather than the systemic RAS, likely plays a role in the renal excretory effect of perinatal taurine depletion. Although captopril treatment in the present dose can abolish the effect of RAS overactivity on baroreflex sensitivity and autonomic nerve activity in adult female rats that are perinatally depleted of taurine followed by high sugar intake (Thaeomor et al. 2010) and markedly prevent hypertension in SHR (Wyss et al. 1994), it might not completely block intrarenal ACE. In addition, the intrarenal RAS effect of perinatal taurine depletion may act through ACE2 pathways and affects angiotensin (1–7) rather than angiotensin II action. Angiotensin (1–7) is converted from angiotensin I and angiotensin II by ACE2 and acts via Mas receptor to increase renal excretory capacity, i.e., opposite to angiotensin II acting on AT_1 receptors (Carey and Padia 2013; Moon 2013). The key enzyme, ACE2, is not inhibited by captopril. In addition, blocking ACE results in high angiotensin I levels, which will be converted primarily to angiotensin (1–7) by ACE2. These complex intrarenal RAS mechanisms may obscure the effect of captopril in the effect of perinatal taurine depletion on renal function via the RAS.

5 Conclusion

Perinatal taurine exposure affects arterial pressure and renal function, and these effects are amplified by high sugar intake. In adult female rats perinatally depleted of taurine, baroreflex and autonomic dysfunctions are normalized by a short-term inhibition of ACEI. Although high sugar intake after weaning alters renal function before hypertension and insulin resistance development in adult male rats, the same intake fails to alter renal excretory capacity in adult female rats perinatally depleted of taurine. This suggests that sugar intake plays an early role in renal function and a later role (in adults) in arterial pressure control, but does not appreciably alter renal function in the adult, perinatally taurine depleted female rats. Further, these studies suggest that the effects of perinatal taurine depletion on the adult kidney are not primarily regulated by the RAS.

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Part VI
Taurine and Nutrition: The Role of Taurine Supplements

Fructose Feeding Changes Taurine Homeostasis in Wistar Rats

Lea Hüche Larsen, Laura Kofoed Hvidsten Ørstrup, Svend Høime Hansen, Niels Grunnet, Bjørn Quistorff, and Ole Hartvig Mortensen

Abbreviations

ADO	Cysteamine dioxygenase
CDO	Cysteine dioxygenase
CSAD	Cysteinesulfinic acid decarboxylase
EDL	Extensor digitorum longus
HDL	High density lipoprotein
LDL	Low density lipoprotein
NEFA	Non-esterified fatty acids
NS	Non-significant
TauT	Taurine transporter

1 Introduction

Regular food constituents have a profound effect on both development and protection against obesity, type 2 diabetes and other adverse metabolic changes. In the last few decades, dietary fructose has become a major constituent of the modern western diet, with the main source being sucrose from beet- or sugar cane, high fructose corn syrup, fruits, and honey (Tappy et al. 2010).

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In human studies, high fructose intake has been shown to induce dyslipidemia and other adverse metabolic changes (Bantle et al. 2000; Crapo and Kolterman 1984). Many studies have reported that a high fructose diet in rodents causes insulin resistance, dyslipidemia and type 2 diabetes (Basciano et al. 2005; Samuel 2011; Tappy et al. 2010). For that reason a high-fructose diet in rodents has become a common model for the development of dyslipidemia, insulin resistance and type 2 diabetes (Tran et al. 2009). Dietary fructose is therefore believed to be a contributing factor for the marked increase in the incidence of the metabolic syndrome in western countries.

Taurine is a semi-essential β-amino acid and has emerged as a participant in the complex network constituting the pathogenesis of the metabolic syndrome and type 2 diabetes. Several studies suggest that during the development of obesity and type 2 diabetes, dysregulation of taurine homeostasis occurs in the organism. Indeed, a high-fructose diet in rodents has been reported to decrease plasma and liver taurine levels. Furthermore, several animal studies have shown that taurine may prevent fructose induced insulin resistance (El Mesallamy et al. 2010; Nandhini and Anuradha 2002; Nandhini et al. 2004).

In the body, taurine is found in relatively high concentrations in tissues (5–50 mM) but about 3 orders of magnitude lower in plasma (10–100 μM) (Hansen 2001; Huxtable 1992). It is known to have a number of physiological functions, such as conjugation with bile acids, intracellular osmolyte for volume regulation and antioxidant properties (Hansen 2001). Taurine homeostasis in the body involves a balance between the rate of biosynthesis from methionine and cysteine, taurine transport, dietary intake and kidney reabsorption/excretion.

Taurine is synthesized from methionine and cysteine in the liver by two different pathways. In the transsulfuration pathway, which is believed to be the primary pathway of taurine biosynthesis, homocysteine is initially converted to cysteine. Cysteine is then oxidized by cysteine dioxygenase (CDO) to generate cysteinesulfinate, which is further decarboxylated by cysteinesulfinic acid decarboxylase (CSAD) forming hypotaurine. CSAD is believed to be the rate-limiting step in the primary pathway. The secondary pathway involves the oxidation of cysteamine by cysteamine dioxygenase (ADO) resulting in hypotaurine. The hypotaurine generated by both pathways is thought to be spontaneously oxidized to taurine (Simmons et al. 2006; Stipanuk and Dominy 2006; Stipanuk et al. 2006; Ueki et al. 2012).

The taurine transporter (TauT) is thought to be expressed throughout the body, and belongs to a family of Na^+ Cl^--dependent transporters. Taurine uptake across the brush-border membrane of the small intestine is mediated via TauT and H^+/amino acid transporter 1 (Anderson et al. 2009). Recent studies have found that the transport of taurine may be upregulated by inflammation (Mochizuki et al. 2004) and decreased by type 2 diabetes (Merheb et al. 2007). In the blood stream taurine is distributed to tissues and cells where it is taken up by TauT and H^+/amino acid transporter 1. Knocking out the TauT causes greater than a 90 % reduction in taurine content in some tissues, thereby demonstrating that TauT is the main uptake transporter for taurine (Heller-Stilb et al. 2002). Skeletal muscle accounts for more that 70 % of the total taurine content in the body (Huxtable 1992) and knocking out the

TauT leads to skeletal muscle impairment (Warskulat et al. 2004). Taurine excretion is either by the kidney through urine or through taurine-conjugated bile acid excretion in feces (Glass et al. 1992; Odle et al. 1992)

The effects of high fructose intake on taurine content and its metabolism are largely unknown. Thus, we examined the effects of high-fructose diet (60 % energy from fructose) with or without oral taurine supplementation (2 % in drinking water) for 6 weeks on taurine homeostasis in Wistar rats.

2 Methods

2.1 *Animals, Study Design and Diet*

All experimental procedures described were approved by The Danish Animal Experiments Inspectorate (permit 2013-15-2934-00904) and by the local animal facility at the University of Copenhagen, Denmark. Forty-eight 8-week old male Wistar Hannover GALAS (HanTac:WH) rats were purchased from Taconic (Ejby, Denmark). Food- and water intake were measured bi-weekly. After acclimatization for 1 week, the rats were randomly divided into four groups ($n = 12$ per group). The animals were fed a control diet or a high fructose diet with or without 2 % taurine supplementation in the drinking water for 6 weeks. The control diet contained 67.3 % energy from carbohydrate (split evenly between corn starch and sucrose), 20 % protein, 12.8 % corn oil. The fructose-rich diet contained 66.8 % energy from fructose, 20.2 % protein, and 12.9 % energy from lard (Harlan Teklad). The taurine used was a chemically synthesized variant (Sigma-Aldrich) and was dissolved directly in the water used in the animal facility. The rats were fed ad libitum, housed two rats per cage, and kept at a 12-h light/dark cycle.

Overnight fasted rats were sedated with a mixture of Hypnorm (active ingredients fentanyl and fluanisone at a concentration of 0.079 mg/ml and 2.5 mg/ml, respectively) and Dormicum (active ingredient midazolam at a concentration of 1.25 mg/ml) in water given as 0.3 ml per 100 g of body weight. Soleus and EDL muscles were dissected from the legs, and the rat was cut open and the liver lobes were dissected. All tissues were quick-frozen in liquid nitrogen and stored at −80 °C for further analysis.

2.2 *Quantitative Real-Time PCR*

RNA was extracted from different tissues using Qiazol (Qiagen, Valencia, CA, USA) as described earlier (Larsen et al. 2013). Total RNA was mixed (at a concentration > 0.15 μg/μl for a total of 2 μg RNA in 20 μl volume) with reverse transcriptase, random hexamer primers and nucleotides and cDNA synthesis performed using the High Capacity cDNA Reverse Transcription Kit with RNase inhibitor (Applied Biosystems, Carlsbad, CA, USA) as described earlier (Larsen et al. 2013)

Amplification mixtures were amplified using a SYBR Green mastermix (Applied Biosystems) according to standard conditions ((95 °C 10 min) × 1, (95 °C 15 s, 60 °C 1 min, 95 °C 15 s, 60 °C 15 s, 95 °C 15 s) × 50 cycles in a total volume of 10 μl with a melting curve from 60 to 100 °C) in 384 well plates in triplicates on an ABI VIIA7 real-time PCR system (Applied Biosystems). TBP mRNA levels were used for normalization between samples. The sequences of primers used are:

Primers:
TauT-forward: 5′-TGGACAGCCAGTTTGTTGAAG-3′,
TauT-reverse: 5′-GCAATGAAGATTTCCCGACGA-3′,
CSAD-forward: 5′-TGGTCATGGAGCCCAAGTTC-3′,
CSAD-reverse: 5′-CATCATGGTTCCCTTCTTCACC-3′,
CDO-forward: 5′-GCCTTCACTTGTACAGTCCAC-3′,
CDO-reverse: 5′-CTCCAGTGAACCTGAAGTTGTAAAT-3′,
ADO-forward: 5′-CCGGTCACTTACATGCACATC-3′,
ADO-reverse: 5′-CGTACAGCACCTTGAGCATAC-3′,
TBP-forward: 5′-CCCACCAGCAGTTCAGTA-3′,
TBP-reverse: 5′-CAATTCTGGGTTTGATCATTC-3′.

2.3 Biochemical Analysis

Fasting glucose was measured using two different automated Accu-Check Glucometers (Roche, Basal, Switzerland) in duplicate from tail vein blood.

Triglycerides were measured in 50 mg liver tissue, hydrolyzed in 0.5 M KOH/85 % ethanol at 60 °C for 30 min. After cooling, $MgSO_4$ was added to 0.1 M and samples were vortexed and centrifuged at 16,000 × *g* for 20 min at 4 °C. Glycerol was measured spectrophotometrically at 340 nm as described (Wieland 1984). High Density Lipoprotein (HDL) cholesterol and Low Density Lipoprotein (LDL) cholesterol in rat plasma were measured at 450 nm using an ELISA Kit according to instructions from the manufacturer (Novatein Biosciences, Cambridge, MA, USA) at 37 °C. Total cholesterol was calculated as HDL + LDL. Plasma Non-Esterified Fatty Acids (NEFA) were measured at 546 nm using NEFA-HR (2) Kit according to instructions from the manufacturer (WAKO, Richmond, VA, USA) at 37 °C.

Taurine content were measured in 10 μl plasma or 50 mg liver-, EDL- or soleus tissue, by homogenization in 10 % (w/v) TCA, followed by neutralization with 1 M KOH in 100 mM Imidazole buffer. Taurine content was measured spectrophotometrically as described (Matsuda and Asano 2012).

2.4 Statistic Analysis

Data are presented as means ± standard error of the mean (SEM). Statistical analyses were carried out using mixed model two-way ANOVA. The mRNA data were log-transformed before statistical analysis in order to obtain a normal distribution except

for EDL ADO mRNA data that had a normal distribution without log-transformation. All statistical analyses were performed using SAS 9.2 (The SAS Institute, Cary, NC, USA). A p-value less than 0.05 was considered significant and a p-value below 0.1 was considered a tendency. A p-value above 0.1 was considered non-significant (NS).

3 Results

3.1 Body Weight, Food Intake, Water Intake and Taurine Intake

All animals demonstrated a steady weight gain (data not shown). Fructose-fed animals had an increase in food intake, but also an increase in water intake (Table 1).

Due to increased water intake the fructose-fed animals consumed significantly more taurine than the controls during all six weeks (Table 1). Despite the increase in calorie intake the fructose-fed animals had no difference in body weight after 42 days compared to the controls. Taurine supplementation had no effect on body weight, food intake or water intake (Table 1).

3.2 Plasma Parameters

Fructose increased plasma triglyceride content, but interestingly, fructose also caused a significant decrease in hepatic triglyceride content (Table 2). However, the fructose diet had no effect on the levels of total cholesterol, HDL, LDL, FFA or fasting glucose. Taurine supplementation did not rescue the plasma parameters or the hepatic changes. However, taurine caused a significant increase in fasting glucose (Table 2).

Table 1 Body weight, food intake, water intake and taurine intake

	Group				Two-way ANOVA		
Parameter	Con	Con + tau	Fru	Fru + tau	Diet	Tau	Int
BW day 1 (g)	280 ± 4	282 ± 3	281 ± 4	281 ± 4.0	NS	NS	NS
BW day 42 (g)	398 ± 9	400 ± 9	392 ± 11	395 ± 9.6	NS	NS	NS
Food intake (kcal)	2912 ± 85	2883 ± 85	3143 ± 98	3168 ± 104	0.01	NS	NS
Water intake (ml)	1263 ± 118	1389 ± 63	1559 ± 80	1634 ± 68	0.004	NS	NS
Taurine intake (g)	–	27.8 ± 1.3	–	32.7 ± 1.4	0.02	–	–

Male Wistar rats, N = 12 per group, were subjected to four different diet regimes for 6 weeks as described in methods. (Con) Control, (tau) Taurine, (fru) Fructose, (int) Diet * taurine interaction. (BW) Body weight

Table 2 Plasma and liver parameters

Parameter	Group				Two-way ANOVA		
	Con	Con + tau	Fru	Fru + tau	Diet	Tau	Int
FG day 1 (mM)	4.6 ± 0.1	4.8 ± 0.1	4.5 ± 0.1	4.4 ± 0.1	NS	NS	NS
FG day 42 (mM)	4.9 ± 0.1	5.1 ± 0.1	4.9 ± 0.2	5.3 ± 0.1	NS	0.02	NS
Chol (mg/dl)	51.5 ± 2.1	53.2 ± 1.4	51.9 ± 2.0	47.4 ± 1.4	NS	NS	NS
LDL (mg/dl)	24.0 ± 2.0	24.4 ± 0.9	22.7 ± 1.5	22.8 ± 1.5	NS	NS	NS
HDL (mg/dl)	28.3 ± 0.8	29.2 ± 0.9	29.2 ± 1.5	25.3 ± 0.6	NS	NS	NS
NEFA (mmol/l)	1.2 ± 0.1	1.3 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	NS	NS	NS
p-TG (mmol/l)	0.7 ± 0.1	0.7 ± 0.1	1.2 ± 0.1	1.0 ± 0.1	0.001	NS	NS
L-TG (μmol/g)	65.3 ± 3.2	64.2 ± 5.7	43.9 ± 3.7	50 ± 4.3	0.001	NS	NS

Male Wistar rats, N = 12 per group, were subjected to four different diet regimes for 6 weeks as described in methods. (Con) Control, (tau) Taurine, (fru) Fructose, (int) Diet * taurine interaction. (FG) fasting glucose, (chol) Cholesterol, (LDL) Low-density cholesterol, (HDL) High-density cholesterol, (NEFA) Non-esterified fatty acids, (p-TG) Plasma triglyceride concentration, (L-TG) Liver triglyceride concentration

3.3 Taurine Content in Plasma, Liver and Skeletal Muscle

Taurine content in plasma was not affected by fructose. Fructose diet had an opposite effect on liver and muscle, causing increased content of taurine in EDL and soleus muscle and a significant decrease in the hepatic taurine content. Taurine supplementation increased the taurine content in both plasma and skeletal muscle, and surprisingly, taurine supplementation also had a tendency to decrease hepatic taurine content (Fig. 1).

3.4 Taurine Homeostasis in Liver and Skeletal Muscle

In the liver, fructose significantly increased the level of TauT mRNA and had no effect on ADO, CDO or CSAD mRNA levels. Taurine supplementation also increased the hepatic level of TauT mRNA and decreased the level of CSAD mRNA whereas the mRNA levels of ADO and CDO did not change (Table 3).

The fructose diet had different effects on the taurine synthetic enzymes in the two different skeletal muscles. TauT mRNA levels were decreased in EDL but no effect was seen in soleus with the fructose diet. Fructose decreased the mRNA levels of ADO in soleus muscle and had no effect on ADO mRNA levels in EDL. Both CSAD and CDO mRNA levels were significantly decreased in the in EDL muscle of the fructose fed animals whereas no changes were seen on mRNA levels of CSAD and CDO in soleus muscle (Table 3).

Taurine supplementation significantly decreased the levels of TauT and CSAD mRNA levels in soleus muscle. Taurine also caused a decrease in TauT mRNA levels in EDL muscle. Furthermore, Taurine supplementation of the control fed animals caused a significant decrease in the levels of CDO in soleus muscle and a significant increase in ADO mRNA levels in EDL (Table 3).

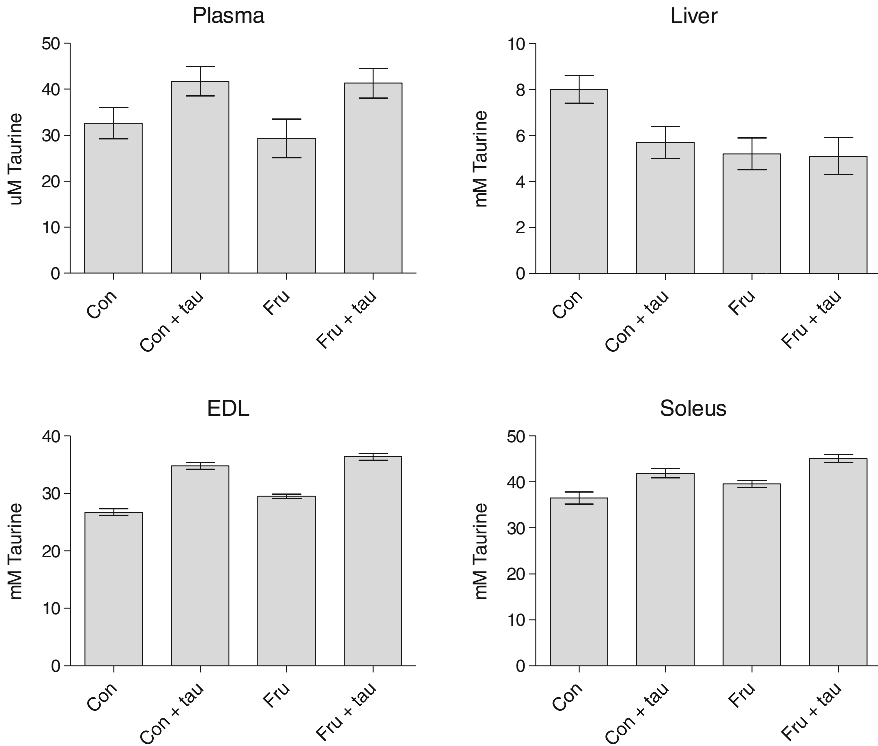

Fig. 1 Taurine content in plasma, liver, soleus and EDL. Male Wistar rats (N = 12) per. group, were subjected to four different diet regimes for 6 weeks as described in methods. Taurine contents were measured as described in methods. (Con) Control, (tau) Taurine, (fru) Fructose. Two-way ANOVA statistics: Plasma; diet: NS, taurine: $p = 0.0041$, diet*taurine: NS. Liver; diet: $p = 0.023$, taurine: $p = 0.09$, diet*taurine: NS. EDL; diet: $p = 0.0003$, taurine: $p < 0.0001$, diet*taurine: NS. Soleus; diet: $p = 0.003$, taurine: $p < 0.0001$, diet*taurine: NS

4 Discussion

The current study set out to investigate the effects of a high-fructose diet on taurine homeostasis in liver and skeletal muscle of Wistar rats. We saw that the fructose diet affected both taurine content and mRNA levels of taurine synthesizing enzymes in liver and skeletal muscle in a tissue specific manner.

4.1 Body and Plasma Parameters

In this study, there was no difference in weight gain between the experimental groups after 6 weeks of dietary manipulation despite an increased calorie intake in the fructose fed group (Table 1). In addition, an increase in water intake in the

Table 3 Liver and skeletal muscles mRNA content

Parameter and tissue	Group				Two-way ANOVA		
	Con	Con + tau	Fru	Fru + tau	Diet	Tau	Int
Liver							
TauT	1.0 ± 0.3	2.9 ± 0.6	1.5 ± 0.4	6.6 ± 0.6	0.003	0.001	NS
ADO	1.0 ± 2.1	0.9 ± 0.1	0.9 ± 0.1	1.1 ± 0.1	NS	NS	0.07
CSAD	1.0 ± 0.2	0.6 ± 0.1	1.0 ± 0.1	0.7 ± 0.1	NS	0.002	NS
CDO	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.0	NS	NS	NS
EDL							
TauT	1.0 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.03	0.04	NS
ADO	1.0 ± 0.1	1.7 ± 0.2	1.0 ± 0.1	0.9 ± 0.1	0.014	0.03	0.016
CSAD	1.0 ± 0.1	1.0 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.002	NS	NS
CDO	1.0 ± 0.2	1.2 ± 0.2	0.5 ± 0.1	0.5 ± 0.0	0.001	NS	NS
Soleus							
TauT	1.0 ± 0.1	0.8 ± 0.1	1.0 ± 0.1	0.8 ± 0.0	0.037	NS	NS
ADO	1.0 ± 0.1	1.2 ± 0.1	0.7 ± 0.1	0.9 ± 0.1	0.003	NS	NS
CSAD	1.0 ± 0.1	0.8 ± 0.1	0.8 ± 0.0	0.8 ± 0.0	NS	0.001	NS
CDO	1.0 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	NS	NS	0.019

Male Wistar rats (N = 12) per. group, were subjected to four different diet regimes for 6 weeks as described in methods. (Con) Control, (tau) Taurine, (fru) Fructose, (int) Diet * taurine interaction, (TauT) Taurine transporter, (CSAD) Cysteinesulfinic acid decarboxylase, (CDO) Cysteine dioxygenase, (ADO) Cysteamine dioxygenase

fructose group was observed, causing the animals in that group to ingest more taurine compared to the control (Table 1), which may explain some of the experimental differences seen in mRNA levels between the taurine fed, control and taurine fed, fructose fed animals as the fructose + taurine animals had a higher taurine intake than the control + fructose animals.

Fructose did not affect fasting glucose levels after 6 weeks of dietary manipulation as shown in several other studies (Bantle 2009). However, taurine supplementation alone significantly increased fasting glucose levels (Table 2), which is inconsistent with previous findings showing that taurine improves insulin sensitivity and reduces hyperglycemia (Nandhini and Anuradha 2003; Nandhini et al. 2005a). Recently we reported an increase in fasting glucose after 26 weeks of taurine supplementation in male Wistar rats (Larsen et al. 2013). Sub-strain differences in the outbred Wistar rat strain is a possible explanation, as there seem to be some rat strain differences in the response to fructose (Stark et al. 2000). The Wistar sub-strain used in the current study only displayed small, if any, effect of fructose compared to other Wistar sub-strains which become severely glucose intolerant after being fed fructose (El Mesallamy et al. 2010; Nandhini et al. 2005a; Perret et al. 2007).

Plasma parameters are often used to identify the general health condition in mammals. Studies have demonstrated increased plasma concentrations of triglycerides and cholesterol in rats subjected to a high-fructose diet (Nagai et al. 2009), (Ackerman et al. 2005). However, in this study, no differences were observed in blood cholesterol, LDL or HDL (Table 2), but consistent with the literature, the

fructose diet increased plasma triglyceride concentrations (Table 2). Taurine did not significantly attenuates fructose-mediated elevations in plasma triglycerides, which seems inconsistent with previous findings which reported a taurine induced attenuation in dyslipidemia and diabetic complications induced by both fructose and high fat ingestion (Murakami et al. 2000; Sethupathy et al. 2002).

4.2 Taurine Content

Studies have shown that taurine content in plasma and in different tissues is affected by different pathological conditions and also the enzymes involved in taurine synthesis and homeostasis are primarily believed to be regulated by diet (Yamamoto et al. 1995). In humans, the amount of taurine in plasma is lowered by obesity, Type 1 diabetes, and type 2 diabetes (Franconi et al. 1995; Jeevanandam et al. 1991; De Luca et al. 2001). Furthermore, high-fat diet-induced obesity in mice presented with decreased plasma taurine content (Tsuboyama-Kasaoka et al. 2006). In the current study, we found that fructose did not affect plasma taurine content which is in direct contradiction with an earlier study, reporting that Wistar rats had decreased taurine content in plasma as a consequence of a fructose diet (Nandhini et al. 2005a).

Moreover, alloxan-induced type 1 diabetic rats display elevated taurine levels in skeletal muscle, and decreased taurine levels in liver (Reibel et al. 1979). Wijekoon et al. found that the ZDF diabetic rat, a model for type 2 diabetes, display increased taurine content in both skeletal muscle and liver (Wijekoon et al. 2004). This indicates that different models affect taurine homeostasis in different ways. We also found an increase in taurine content in skeletal muscle induced by both fructose and taurine. However, in the liver fructose significantly decreased taurine content with no taurine rescue effect. Surprisingly, taurine supplementation had a tendency to decrease taurine content in the liver. Nandhini et al. have also demonstrated a hepatic decrease in taurine content when subjecting Wistar rats to a high-fructose diet (Nandhini et al. 2005b, 2005c).

4.3 Taurine Transport and Biosynthesis

It has been suggested that during the development of obesity and type 2 diabetes, a dysregulation of taurine homeostasis occurs in the organism, possibly orchestrated by changes in the levels of enzymes involved in taurine transport and synthesis. We therefore measured the mRNA levels of the taurine transporter and the enzymes involved in taurine biosynthesis.

Studies have shown that renal epithelium can adapt to the availability of taurine ingested, as the mRNA level and activity of TauT is responsive to the presence of taurine or precursors of taurine. In this way, the kidney can increase reabsorption or excretion of taurine in response to dietary intake (Chesney et al. 1989). We show in

this study that fructose-fed Wistar rats display alterations in mRNA levels of all of the enzymes involved in taurine transport and synthesis. Fructose diet increased mRNA levels of TauT in the liver and suppressed it in EDL muscle. Taurine supplementation had the same effect as fructose on TauT mRNA levels in liver and EDL muscle. In soleus muscle taurine had a suppressing effect, suggesting that dietary fructose is an important regulator of TauT mRNA levels in liver and EDL and a regulator of taurine in all three tissues.

CSAD is believed to be the rate-limiting step in taurine biosynthesis; studies have shown that diet can change CSAD mRNA levels. Jerkins et al. showed that rats fed a high-protein diet contain decreased levels of CSAD mRNA (Jerkins et al. 1998). In the current study, we found a significant decrease in the levels of CSAD mRNA in EDL muscle in the fructose fed animals but no change in soleus muscle and liver. Taurine decreased mRNA levels of CSAD in soleus muscle and liver. This suggests that diet regulates CSAD mRNA levels in EDL muscle and fructose regulates taurine in all three tissues.

A high-fat diet and genetically (db/db) obese mice show decreased mRNA levels of CDO in white adipose tissue (Tsuboyama-Kasaoka et al. 2006). We saw that fructose suppresses CDO mRNA levels in EDL muscle. In the soleus muscle a decrease was observed in mRNA levels of CDO in the control fed animals compared to that of fructose fed animals, which could be due to the difference in taurine intake between the groups. Fructose had no effect on CDO mRNA levels in the liver. This indicates some kind of dietary regulation of CDO mRNA levels in EDL muscle. Furthermore, we showed regulation of ADO by dietary fructose, which is the enzyme involved in what is believed to be the secondary pathway in taurine biosynthesis (Simmons et al. 2006; Stipanuk and Dominy 2006; Stipanuk et al. 2006; Ueki et al. 2012). In the current study fructose decreases the mRNA levels of ADO in soleus whereas taurine supplementation increases the mRNA levels of ADO in EDL in the control fed animals.

5 Conclusion

In the present study, we show for the first time that tissue and plasma taurine is affected by dietary fructose, with the effect greater in liver than in skeletal muscle. Also, the enzymes involved in taurine biosynthesis and the taurine transporter in liver and skeletal muscle are influenced by a fructose diet. These observations give a clear indication that taurine transport and biosynthesis in liver and skeletal muscle may be dysregulated in the fructose fed rat model and possibly also in specific animal models of malnutrition.

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Effects of Taurine Supplementation on Adipose Tissue of Obese Trained Rats

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Abbreviations

PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PPAR	Peroxisome proliferator-activated receptor
SD	Sedentary, without taurine supplentation
SDTAU	Sedentary and taurine supplemented group
TR	Trained group
TRTAU	Trained and taurine supplemented group
WAT	White adipose tissue
WG	Weight gain

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1 Introduction

Although many studies have investigated the best strategy to treat obesity, an exponential increase in the prevalence of this worldwide epidemic has been observed (Azevedo and Brito 2012; Cabeço et al. 2010). Obesity is a multifactorial chronic disease characterized mainly by excessive body fat accumulation. Pêgo-Fernandes et al. (2011) estimated that approximately 1.6 billion adults are classified as overweight and 400 million as obese. In addition, based on the increment of the number of obesity cases, Stothard et al. (2009) demonstrated the severity of this problem to world health. High consumption of hypercaloric diets is one of the responsible factors for the increase in obesity prevalence. In addition, obesity is associated with several chronic diseases such as diabetes, cardiovascular diseases, digestive diseases, respiratory diseases and some types of cancer (Du et al. 2010).

According to Gomes et al. (2012), many studies have used educational, nutritional, pharmacological and surgical strategies, as well as physical exercise programs, for treatment of obesity. The use of specific nutrients, such as taurine supplementation, has been reported as a strategy for reducing body weight (Tsuboyama-Kasaoka et al. 2006). Taurine (2-aminoethanesulfonic acid, Tau) is a non-essential amino acid that can be synthesized from methionine and cysteine. As endogenous production of taurine is insufficient, this amino acid must be provided by the diet, especially by fish and seafood (De la Puerta et al. 2010; Rosa et al. 2014). The association between taurine supplementation and physical exercise is related to the increase in the adiponectin levels and body energy expenditure (Xu et al. 2008), and to the decrease in the adipocyte sizes (Tsuboyama-Kasaoka et al. 2006), in the level of inflammatory markers and in lipid peroxidation (Rosa et al. 2014).

You et al. (2013) used taurine supplementation (3 % in drinking water) to investigate the association of serum taurine levels with serum adiponectin and leptin levels in high-fat diet-induced obese rats. The authors concluded that serum taurine levels were negatively correlated with serum total cholesterol levels and positively correlated with serum adiponectin levels, demonstrating the beneficial effects of taurine supplementation for high-fat diet-induced obesity rats. According to Tsuboyama-Kasaoka et al. (2006), taurine supplementation-induced obesity prevention is associated with an increase in mRNA levels of transcription factors and cofactor involved in energy expenditure, such as PPAR, coactivator (PGC-1α), PPARα, PPARγ, and nuclear respiratory factor 2α in WAT of mice.

Based on the literature, while physical exercise is related to the decrease in body fat mass, taurine supplementation can increase energy expenditure and the function of lipolytic enzymes and may decrease adipocyte size, reflecting an improvement from obesity treatment. However, a possible additive effect of physical exercise and taurine supplementation on WAT has not been investigated. Thus, the aim of this study was to evaluate the effects of taurine supplementation on adipose tissue of trained obese rats.

2 Methods

2.1 Animals and Experimental Protocol

All procedures were reviewed and approved by the Ethics Committee of the University of Sao Paulo (CEUA/USP-RP protocol number 10.1.1290.53.5) in compliance with the US National Institutes of Health Guide Care and Use of Laboratory Animals. Twenty-four male Wistar rats (270–290 g; 8 weeks-old) were fed with a high-fat diet to induce obesity and were divided into four groups: sedentary (SD); sedentary and taurine supplemented (SDTAU); trained (TR); trained and taurine supplemented (TRTAU). Animals were housed in polypropylene cages (41 × 34 × 30 cm), three animals per cage on a 12 h light/dark cycle, and received water or 2 % taurine in water ad libitum (Kim et al. 2012). The high-fat diet contained 16.8 kJ/g and was composed of 56 % carbohydrate, 18 % protein and 26 % fat. The study lasted 11 weeks, and taurine supplementation and/or the exercise program started 3 weeks after the beginning of the study. Animals were weighted weekly, and weight gain (WG = final weight − initial weight) during the supplementation period was evaluated considering initial weight (before taurine supplementation) and final weight (after supplementation).

2.2 Exercise Program

Prior to the beginning of exercise training, rats were adapted to treadmill running for 4 days. This period consisted of daily running sessions starting at 5 m min^{-1} on the first 2 days and 10 m min^{-1} on the third and fourth day. The pre-training adaptation period lasted 30 min on first and third days and 60 min on second and fourth days. An incremental treadmill test, adapted from Hohl et al. (2009), was performed to determine the exhaustion velocity (considered as the maximal velocity). The test began with animals running at 11.6 m min^{-1}, with an incremental increase of 1.6 m min^{-1} every 2 min until 20 m min^{-1}, when the speed was increased 3.2 m min^{-1} every 2 min. Exhaustion was determined when rats failed to maintain a running speed adequate to avoid touching the treadmill grid five times in 1 min. The exhaustion speed was considered as the maximal velocity (mv) required to establish training speed running. Exercise training consisted of continuous treadmill running for 60 min, 5 days per week, for 8 weeks. Each training week consisted of 5 days of training followed by 2 days of recovery. Training intensity was progressively increased, starting at 40 $\%_{mv}$ at the first week, 50–55 $\%_{mv}$ from second to fourth week, and 60 $\%_{mv}$ from fifth to eighth week of training.

2.3 Computed Tomography Exam

In the last training week, the experimental animals were submitted to computed tomography of the abdomen, which comprised the height of the diaphragmatic surface to the level of L4-L5 vertebrae, heart, ventricular middle third, and liver, the right lobe. The equipment used was a helical Computed Tomography scanner (Emotion, Siemens, Erlangen, Germany). Images were recorded in ventral decubitus position with the rat fasted for 12 h. The images were analyzed using MATLAB, version 7.0 (Graphics Software, Natick, Massachusetts, USA).

2.4 Sample Collection

Rats were anaesthetized 48 h after the last exercise session (i.e. at the end of week 11). After an overnight fast (~12 h), rodents were anaesthetized with an intraperitoneal (i.p.) injection of thiopental (35 mg kg^{-1} of body weight) and euthanized by decapitation. The epididymal and perirenal adipose tissues were extracted, weighed and used for histological analysis.

2.5 Histological Analysis of Adipocytes

Perirenal adipose tissue was cut into slices and immersed in 10 % buffered formalin to be cut and dehydrated by immersion in alcohol of increasing concentration. Subsequently, the samples were immersed in 110 % xylol bath and submerged in the liquid paraffin bath, which lead to embedding of the samples in paraffin blocks. The blocks were cut and mounted on slides, stained with Harris hematoxylin and counter stained with Eosin Phloxine solution. For subsequent hydration, the blade was immersed in increasing concentrations of alcohol and then cleared in xylem which is miscible with Entelan® (Merck & Co. Inc, AS) used for mounting the blades. The images were analyzed using the "Image J", version 1.34 (Wayne Rasband, National Institutes of Health, Bethesda, MD).

2.6 Statistical Analysis

Results were presented as means plus or minus standard error of the mean. One-way Analysis of Variance (ANOVA) was performed with Tukey-Kramer post test for analysis of different groups using SPSS software, version 15.0. The level of significance for all comparisons was set at $p \leq 0.05$.

3 Results

Physical exercise training improved the exercise capacity of the trained rats, about 45 % from 4th to 11th week in a similar manner (from 127 ± 9 to 184 ± 7 kg/m for TR group and 112 ± 11 to 172 ± 19 kg/m for TRTAU group). Table 1 shows that TR and TRTAU showed a significantly lower body weight compared to that of the SD and SDTAU groups (Table 1). In addition, the SDTAU group exhibited a higher water intake compared to that of the SD and TR group.

Table 1 shows that SDTAU and TRTAU contained higher serum taurine values compared to that of the TR group. In relation to total body area and visceral fat area, TRTAU presented lower values compared to the SDTAU group. In fact, the difference in total body area between these groups was about 26 % ($p = 0.007$). In addition, both TR and TRTAU contained lower epididymal fat than SD and SDTAU (Table 2). Figure 1 show that adipocyte sizes were not different between the experimental groups.

Table 1 Initial weight, weight gain, food consumption, water intake and serum taurine concentration of the studied groups

Experimental Groups	SD	SDTAU	TR	TRTAU
Initial weight (g)	246 ± 3.4	247 ± 4.7	243 ± 2.3	237 ± 2.2
WG (g)	498 ± 24	540 ± 27	403 ± 18[a,b]	398 ± 27.5[a,b]
Daily food intake (g/d)	30.6 ± 2.4	30.6 ± 1.3	26.6 ± 3.0	30.6 ± 2.3
Water intake (mL/d)	43.5 ± 1.0	50.5 ± 1.8[a,c]	44 ± 1.1	47 ± 1.8
Serum taurine concentration	279.26 ± 41.2	358.4 ± 27.3	185.1 ± 15.7[b]	338.7 ± 32.2[c]

Values are expressed as means ± SE of n = 6. WG (weight gain) = final weight − initial weight. Statistical differences between the groups were detected using ANOVA, ($p < 0.05$)

[a]Statistical differences in relation to group SD

[b]Statistical differences in relation to group SDTAU

[c]Statistic difference in relation to group TR

Table 2 Total body area, visceral fat area, epididymal fat and adipocyte sizes of the studied groups

Experimental Groups	SD	SDTAU	TR	TRTAU
Total body area (cm^2)	34.8 ± 2.1	34.8 ± 2.1	29.7 ± 1.5	26.4 ± 1.8[a]
Visceral fat area (cm^2)	17.1 ± 3.4	23.0 ± 2.1	11.2 ± 1.7	7.9 ± 1.4[a]
Epididymal fat (g)	23.0 ± 1.6	20.3 ± 2.0	13.4 ± 0.9[a,b]	12 ± 1.0[a,b]
Adipocyte sizes (pixels)	12,017 ± 1,198	10,970 ± 4,233	10,136 ± 1,235	7,763 ± 2,162

Values are expressed as means ± SE of n = 6. Statistical differences between the groups were detected using ANOVA, ($p < 0.05$)

[a]Statistical differences in relation to group SDTAU

[b]Statistical differences in relation to group SD

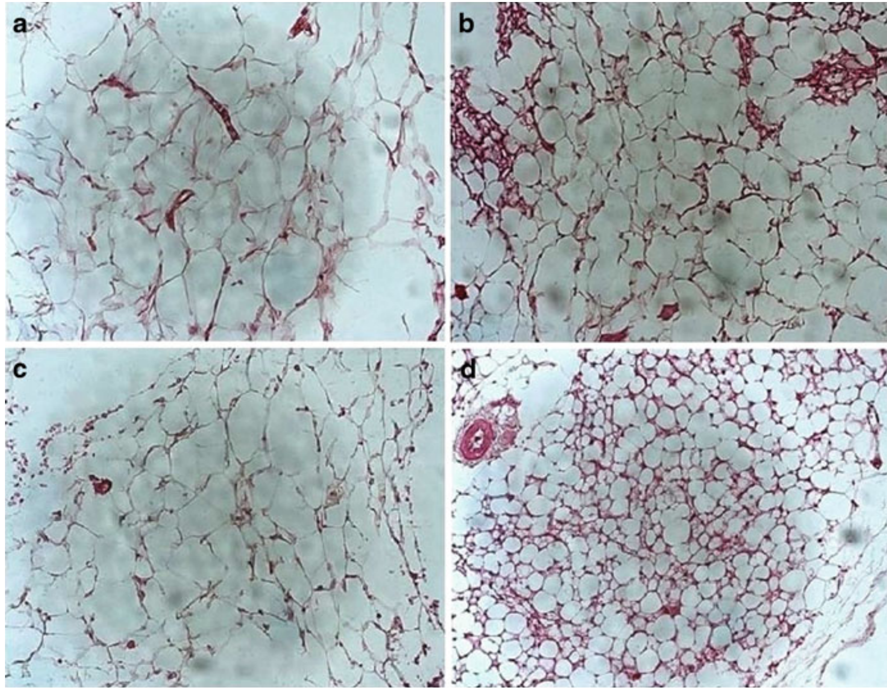

Fig. 1 Image of the histology of the adipocyte. Sedentary group—SD (**a**), sedentary with supplementation group—SDTAU (**b**); trained group—TR (**c**); trained with supplementation group—TRTAU (**d**)

4 Discussion

The present study evaluated the effects of taurine supplementation on adipose tissue of trained obese rats. The main findings of this study are: (a) the high-fat diet increased body weight of the studied groups; however, the TRTAU group exhibited a lower final body weight; (b) the combination of physical exercise and taurine supplementation (TRTAU group) decreased fat area and epididymal fat weight.

As previously stated, the groups supplemented with taurine (i.e. SDTAU and TRTAU) showed higher blood taurine concentration compared to the TR group. Rosa et al. (2014) showed that obese women supplemented with taurine for 8 weeks increased their serum taurine concentration approximately 97 % compared to the placebo group. The high-fat diet used in the present study effectively increased body weight of all experimental groups (i.e. SD = 302.2 %; SDTAU = 318.8 %; TR = 266.1 %; TRTAU = 255.3 %). It is important to point out that TRTAU had a lower body weight gain than the other groups. Similar results were found by Gomes et al. (2012), who compared obese, exercised with obese, non-exercised rats.

According to Mauad (2011), the use of computed tomography is considered the gold standard for the determination and quantification of abdominal fat compartments.

Makrogiannis et al. (2013) confirmed the reliability of this method and reinforced the view that a computed tomography scan is a widely adopted technique to assess abdominal fat. The present data compared visceral fat area by computed tomography; we found that significant differences exist between SDTAU and TRTAU. This result proved that a reduction of visceral fat is possible only when physical exercise and taurine supplementation are combined. According to Tsuboyama-Kasaoka et al. (2006), taurine supplementation increases the mRNA levels of transcription factors and cofactor involved in energy expenditure, such as peroxisome proliferator-activated receptor (PPAR), coactivator (PGC-1α), PPARα, PPARγ, and nuclear respiratory factor 2α in white adipose tissue (WAT) of mice. In a related study, Kwon et al. (2010) verified that a program of resistance exercise performed at low intensity was unable to reduce computed tomography-measured visceral fat in diabetic women.

Regarding the selected exercise program, both groups (i.e. TR and TRTAU) improved their performance; however, no additional benefits were observed with taurine supplementation. In addition, it is important to note that exercise improved body composition once TR and TRTAU showed significant differences in the weight of epididymal fat compared to the sedentary group. Chung et al (2013) observed a significant reduction of 21 % in epididymal fat of obese trained animals. The authors also evaluated the effect of Rubus coreanus extract and concluded that physical exercise alone plays an important role in epididymal fat reduction, but when it was combined with certain substances, this effect can be potentiated. In another study, Du et al (2010) observed a reduction in epididymal fat of obese rats that received a hyperlipidic diet that was supplemented with lotus leaf extract and taurine. The authors concluded that the supplements showed anti-obesity and hypolipidemic effects in obese rats.

Besides the benefit of taurine supplementation in body composition, some authors have already reported that taurine may improve blood lipids (Murakami et al. 2000; Harada et al. 2004; Zhang et al. 2004). Taurine enhances the activity of cholesterol 7 alpha-monooxygenase, a rate-limiting enzyme in bile acid synthesis, stimulates cholesterol catabolism and dietary cholesterol absorption. Thus, taurine supplementation attenuates the rise in total cholesterol and fractions in healthy men consuming a high-fat diet (Murakami et al. 2000; Budhram et al. 2013).

5 Conclusion

Based on the current results, we conclude that 8 weeks of taurine supplementation associated with exercise training, is able to reduce visceral fat and decrease the weight of epididymal fat. However, taurine supplementation with and without exercise did not cause significant changes in the size of the adipocytes. Thus, we suggest that taurine supplementation associated to exercise promotes positive effects on adipose tissue and may be used as a strategy to treat obesity.

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The Effect of Taurine Supplementation on Glucose Homeostasis: The Role of Insulin-Degrading Enzyme

Rafael Ludemann Camargo*, Renato Chaves Souto Branco*, Luiz Fernando de Rezende, Jean Francisco Vettorazzi, Patricia Cristine Borck, Antônio Carlos Boschero, and Everardo Magalhães Carneiro

Abbreviations

AKT	Protein kinase B
GSIS	Glucose-stimulated insulin secretion
HFD	High-fat diet
IDE	Insulin-degrading enzyme
Tau	Taurine

1 Introduction

Taurine (Tau) (2-aminoethanesulfonic acid) is a sulfur-containing amino acid that is classified as semi-essential. Tau is not incorporated into proteins; however, this amino acid has been shown to affect a variety of cell types, contributing to the control of cell volume as an osmolyte, the production of specific bile acids and the regulation of intracellular ion homeostasis. For example, Tau regulates Ca^{2+} homeostasis and K_{ATP} channel activity. Moreover, Tau also participates in cytoprotection, oxidative stress and neurotransmission in the central nervous system, the retina, muscle tissue, the pancreas and others (De la Puerta et al. 2010; Ripps and Shen 2012; Huxtable 1992).

Numerous studies have shown that Tau affects glucose homeostasis, mainly through its effects on insulin secretion and signaling. In pancreatic β-cells, Tau

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regulates insulin secretion in response to different nutrients that affect Ca^{2+} and K^{+} currents (Cuttitta et al. 2013; Park et al. 2004). The effects of Tau are also observed in the insulin-signaling cascade of peripheral tissues, such as the liver and skeletal muscle. The supplementation of this amino acid or its intraperitoneal infusion can promote both basal and insulin-stimulated tyrosine phosphorylation of the insulin receptor in mice (Carneiro et al. 2009).

The liver is able to remove approximately 50 % of the secreted insulin after just two passages. Thus, the liver is the major organ responsible for insulin clearance (Duckworth et al. 1998; Boden et al. 1996; Butterfield 1970). On the molecular level, insulin clearance is controlled by the expression and activity of the insulin-degrading enzyme (IDE), a 110-kDa zinc metalloproteinase expressed in insulin-responsive tissues, particularly in the liver. IDE is responsible for the majority of insulin that is removed and/or degraded from the plasma (Duckworth et al. 1998; Authier et al. 1996; Amata et al. 2009; Kuo et al. 1993).

The studies described above provide an accurate overall picture of the effects of Tau supplementation on insulin secretion and sensitivity. However, there are currently no data regarding the effect of Tau on insulin clearance, which is one of the major processes controlling insulinemia and glucose homeostasis (Park et al 2006; Ahrén et al. 2005; Meier et al. 2005; Valera Mora et al. 2003; Mittelman et al. 2000).

Therefore, the aim of the present study was to assess the effect of Tau supplementation on hepatic IDE expression, which is related to insulin clearance in mice.

2 Methods

2.1 Animals and Experimental Design

In this study, weaned 30-day old male C57BL6 mice were obtained from the breeding colony at UNICAMP and maintained at 22 ± 1 °C on a 12 h light-dark cycle with free access to food and water. The mice were randomly distributed into two experimental groups. One group received drinking fresh water with no Tau added (C group) and another received 5 % of Tau supplementation in the drinking water (CT group) for 14 weeks. During the last week, food intake was performed and after this period, the animals were subjected to experimental procedures. All experiments were approved by the State University of Campinas Ethics Committee (protocol number: 2826-1).

2.2 Intraperitoneal Glucose Tolerance Test

Mice received an intraperitoneal injection of glucose (1 g/kg) after 12 h of fasting. Blood samples were collected from the tail immediately before injection and after 15, 30, 60 and 120 min to determine glucose concentrations. Blood glucose was measured by a glucose strip on an Accu-Chek Performa II instrument (Roche, Indianapolis, IN, USA).

2.3 Intraperitoneal Insulin Tolerance Test

Non-fasted mice received an intraperitoneal injection of insulin (1 U/kg). Blood glucose was measured using test strips (Accu-Chek Performa II) at baseline (before insulin administration) and 2, 4, 8, 12, 16, 20, 30 and 60 min after insulin injection. Glucose measurements were then converted to the natural logarithm (Ln). The slope was calculated using linear regression (time × Ln[glucose]) and was multiplied by 100 to obtain the glucose decay constant rate during the insulin tolerance test (k_{ITT}) per minute (%/min).

2.4 Tissue Samples

At the end of experimental period, 12 h fasted mice were euthanized in a CO_2 chamber followed by decapitation. The livers extracted from mice were snap-frozen in liquid nitrogen, and stored at −80 °C for subsequent protein extractions and western blot experiment.

2.5 Western Blot

Protein expression and phosphorylation were evaluated as previously described (Santos et al. 2011).

2.6 Islets Isolation and Static Insulin Secretion

Pancreatic islets were isolated from mice by the collagenase method, as previously described (Rezende et al. 2009). For static incubations, batches of four islets were pre-incubated for 1 h in Krebs-Henseleit buffer solution (KHBS) containing 0.5 g/l BSA and 5.6 mmol/l glucose and equilibrated at 95 % O_2 and 5 % CO_2 at 37 °C. The medium was discarded, and the islets were incubated for an additional 1 h in 1 ml KHBS containing 2.8 or 16.7 mmol/l glucose. Subsequently, the supernatant fraction was collected to evaluate insulin secretion by radioimmunoassay, as previously described (Rezende et al. 2007).

2.7 Statistic Analysis

Point-to-point comparisons were made using Student's *t* test. Groups were also compared by Student's *t* test. The results were considered significantly different if $p < 0.05$.

3 Results

3.1 Mice Features and Food Intake

During the last week of treatment, we observed an 18.2 % reduction in food intake in the CT group compared with the C group ($p<0.05$; Fig. 1a). At the end of the experimental period, the weight of the retroperitoneal fat pad in the CT group exhibited a 21.8 % decrease ($p<0.05$; Fig. 1b), but no changes in body weight (Fig. 1c) or the Lee index (Fig. 1d) were observed.

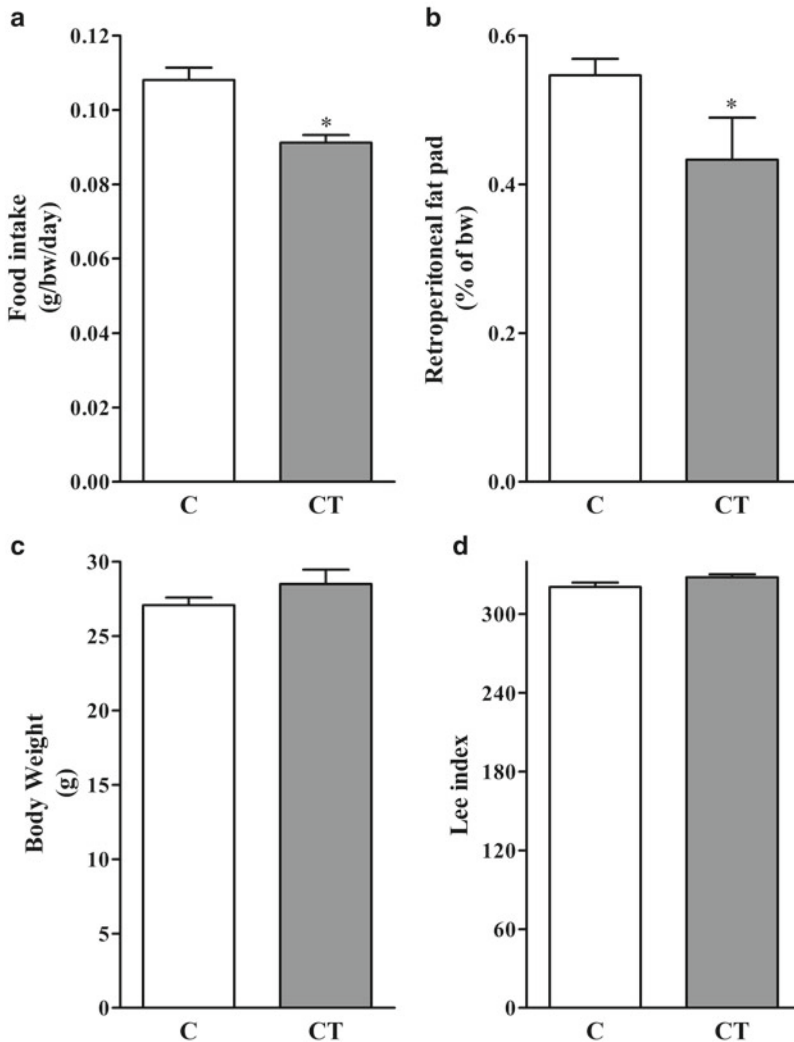

Fig. 1 Food intake (**a**), retroperitoneal fat pad (**b**), total body weight (**c**) and Lee index (**d**) of C and CT mice. The values are the means ± SE, n = 4–15 per group. *Significantly different from C mice (Student's *t* test; $p<0.05$)

3.2 Glucose Tolerance and Insulin Sensitivity

Tau supplementation increased glucose tolerance of the mice, as evidenced by the ipGTT (Fig. 2a) and a 10.7 % reduction in the ipGTT AUC ($p<0.05$; Fig. 2b). Moreover, the CT group also exhibited improved insulin sensitivity as evidenced by the ipITT (Fig. 3a) and a 26.8 % increase in the kITT ($p<0.05$; Fig. 3b).

3.3 Glucose-Stimulated Insulin Secretion (GSIS)

In both groups, we observed an increase in GSIS when the islets were stimulated with 16.7 mmol/L of glucose compared with 2.8 mmol/L of glucose ($P<0.05$, Fig. 4). However, no significant difference in the GSIS was detected between the CT group and the C group in response to stimulation with 16.7 mmol/L of glucose (Fig. 4).

3.4 IDE Expression

To evaluate hepatic IDE expression, we performed western blot of liver tissue samples obtained from fasting mice. Contrary to our results for insulin secretion, Tau supplementation resulted in a 2.3-fold increase in hepatic IDE protein expression ($p<0.05$; Fig. 5).

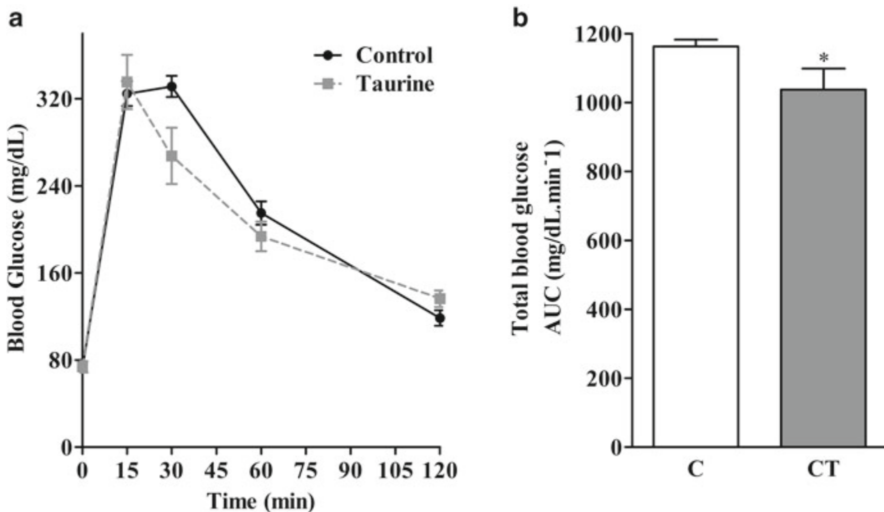

Fig. 2 Glucose tolerance (**a**) and AUC of glucose during the ipGTT (**b**) in C and CT mice fasted for 12 h. Values shown are the means ± SE, n = 8–12 per group. *Significantly different from C mice (Student's *t* test; $p<0.05$)

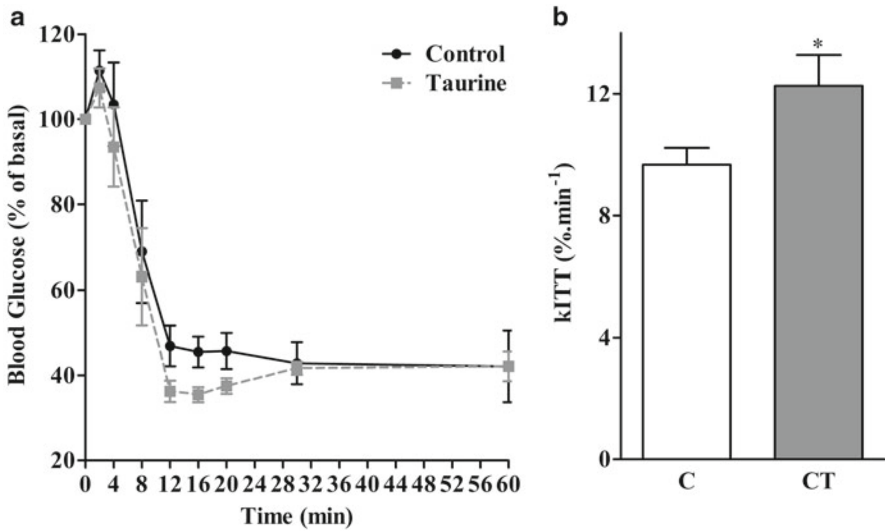

Fig. 3 Insulin tolerance Test (**a**) and kITT (**b**) in non-fasting C and CT mice. Values shown are the means ± SE, n = 4 per group. *Significantly different from C mice (Student's *t* test; $p < 0.05$)

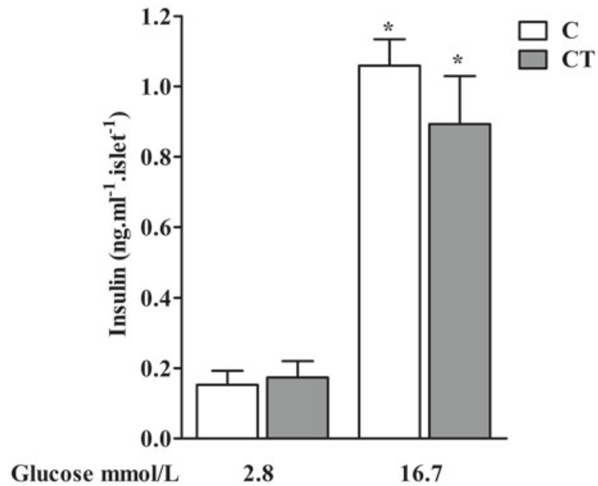

Fig. 4 Glucose-stimulated insulin secretion by pancreatic islets in C and CT mice. Values shown are the means ± SE, n = 12 groups of islets. *Significant differences between C and CT mice with 2.8 and 16.7 mmol/L of glucose. (Student's *t* test; $p < 0.05$)

4 Discussion

Mice supplemented with Tau exhibited lower food intake and reduced fat pad, but no changes in the Lee index or body weight were observed. The mice that received Tau supplementation also presented an increase in glucose tolerance and insulin sensitivity, but there were no changes in insulin secretion by pancreatic islets.

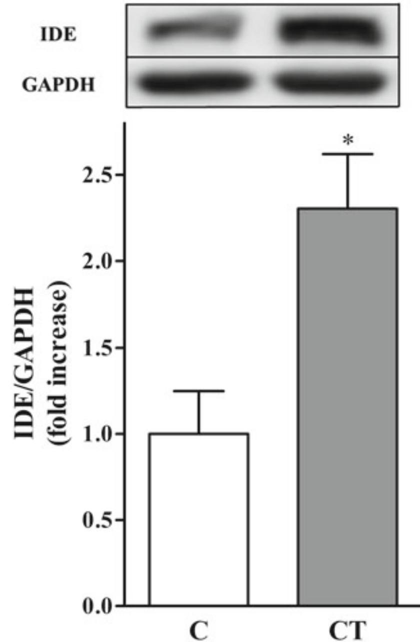

Fig. 5 IDE protein levels in the livers of C and CT mice. Values shown are means ± SE, n = 4 per group. *Significantly different from C mice (Student's *t* test; $p < 0.05$)

Furthermore, Tau supplementation increased hepatic IDE expression, which may indicate a role in insulin clearance.

Tau has been shown to have beneficial effects in pathologic conditions, such as high fat diet (HFD)-induced insulin resistance and streptozotocin-induced diabetes (Trachtman et al. 1995). In obese mice, Tau supplementation decreased body weight, fat pad weight and also resulted in a caloric intake reduction in mice fed a HFD (Batista et al. 2013; Camargo et al. 2013).

Recent studies from our group have demonstrated that Tau supplementation in obese mice induced by an HFD improves glucose tolerance and insulin sensitivity, which are impaired by obesity (Batista et al. 2013; Camargo et al. 2013; Cappelli et al. 2014; Vettorazzi et al 2014). This improvement can be explained by a decrease in insulin hypersecretion (Vettorazzi et al 2014; Ribeiro et al 2012) and an increase in hepatic and muscle AKT phosphorylation (Batista et al. 2013; Ribeiro et al. 2012) seen in obese mice fed a HFD. Female mice supplemented with 5 % Tau in the drinking water, present no difference in glucose tolerance but prevent compensatory morpho-functional adaptations in the islets of mice fed a HFD (Ribeiro et al. 2012). Moreover, 3-month-old female taurine transporter knockout mice do not show any worsening in the glucose tolerance test (Schaffer et al. 2013). Preliminary data from our group show that Tau supplementation in menopausal female mice may worsen the glucose tolerance induced by obesity. Probably there is a difference in peripheral taurine sensitivity on glucose tolerance between female and male mice.

The main role of IDE in insulin-responsive organs is to degrade and/or partially inactivate insulin, clearing insulin from the plasma (Duckworth et al. 1998; Authier et al. 1996; Amata et al. 2009; Kuo et al. 1993). Reducing IDE expression and/or activity has been shown to reduce insulin clearance in many human and animal models (Rezende et al. 2012; Rudovich et al. 2009; Slominskiĭ et al. 2009; Groves et al. 2003). In addition, changes in IDE levels are related to the development of metabolic diseases, such as type 2 diabetes (Farris et al. 2003, 2004; Abdul-Hay et al. 2011).

In protein-restricted mice, a decrease in IDE expression in the liver and muscle was observed, which may explain the improvement in insulin sensitivity, despite a decrease in GSIS (Rezende et al. 2014). On the other hand, female obese mice fed a cafeteria diet exhibited insulin hypersecretion and a decrease in IDE expression in the liver and muscle, which may explain the observed glucose intolerance and the decrease in insulin sensitivity (Brandimarti et al. 2013). In the present study, we observed that Tau supplementation increased hepatic IDE expression in mice with normal metabolism, indicating that supplementation with this amino acid could modulate glucose homeostasis, even without any previous metabolic disruption.

Based on our results, it is premature to conclude that increased IDE expression in the liver contributes to improvement in glucose tolerance, and the role of IDE itself in the development and progression of type 2 diabetes is still controversial (Rezende et al. 2012; Brandimarti et al. 2013; Matveyenko et al. 2008; Erdmann et al. 2008; Goodarzi et al. 2011; Tamaki et al. 2013; Lorenzo et al. 2013). Nevertheless, IDE is certainly a key metabolic enzyme with an important role in the control of glucose homeostasis, and it is affected by Tau supplementation.

5 Conclusion

This study demonstrated that supplementation with taurine in the drinking water resulted in a significant increase in the expression of insulin-degrading enzyme in the liver without affecting insulin secretion. Although the latter has already been described, this is the first evidence indicating that taurine might control glucose homeostasis by altering insulin clearance.

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The Association Among Dietary Taurine Intake, Obesity and Quality of Sleep in Korean Women

Chae Ryung Ha, So Hyun Kim, Su Bin Na, Jeong Soon You, and Kyung Ja Chang

Abbreviations

BMI Body mass index
HDL High density lipoprotein
LDL Low density lipoprotein
SE Standard error

1 Introduction

Obesity is becoming a health problem and is rapidly increasing worldwide (Reilly 2006). Obesity is associated with many health effects, including diabetes, cancer, and cardiovascular disease (Gillis et al. 2002) and is related to sleepiness and fatigue (Vgontzas and Bixler 1998; Seicean et al. 2007). Taurine may play a significant role in physical aspects, such as fatigue recovery, anti-obesity, and antioxidation (Stapleton et al. 1997). Recent findings also imply an association between short sleep duration and weight gain (Hasler et al. 2004). The quantity and quality of sleep is related to health, however, a study on the quality of sleep is not enough. In particular, a study on quality of sleep of obese persons is insufficient. Therefore, the purpose of this study was to investigate the relationship between dietary taurine intake, obesity, and quality of sleep in Korean women.

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2 Method

2.1 Subjects

The 16 subjects were Korean women in the age group of 30–54 years. The subjects were residing in Incheon, Korea. The subjects were investigated for the relationship between taurine intake, blood lipid profiles, and quality of sleep. Age, amount of exercise, marital status, and alcohol drinking were included in questions regarding general characteristics. Height was measured using a stadiometer. Body weight, body mass index (BMI), and percent body fat were assessed using the InBody 720 Composition Analyzer (InBody 720, Biospace, Seoul, Korea). The subjects were divided into three groups according to BMI: Group 1; Normal and overweight (BMI; 18.5–24.9), Group 2; ObesityI (BMI; 25.0–29.9), and Group 3; ObesityII (BMI; over 30).

2.2 Blood Lipid Profiles

Blood total-cholesterol, high density lipoprotein (HDL)-cholesterol, and triglyceride levels were analyzed after 12 h-fasting blood collections. Low density lipoprotein (LDL)-cholesterol was calculated using the Friedewald formula (Friedewald et al. 1972); LDL-cholesterol = Total cholesterol – (Triglyceride/5 + HDL-cholesterol).

2.3 Quality of Sleep Measurement

Quality of sleep score was determined using 'The Korean sleep scale A questionnaire'. Higher sleep scores indicated higher quality of sleep. The sleep score scale contained 15 items. Responses for each item ranged from 1 to 5.

2.4 Dietary Taurine and Major Nutrient Intake Measurement

Dietary taurine and major nutrient intake were surveyed using a 3-day recall method (2 week days and 1 weekend day) and estimated using the CAN-pro 3.0 (The Korean Nutrition Society, Seoul, Korea). The program included a taurine content database of 17 food groups commonly used in 310 food items.

2.5 *Statistical Analysis*

Statistical analysis was performed using the SPSS 21.0 program. Data were expressed as means ± standard error (SE) and N (%). Data were analyzed for significant differences by one-way analysis of variation followed by Duncan's multiple range tests at a $p < 0.05$. Correlation between dietary taurine intake, BMI, and quality of sleep was analyzed using Pearson's correlation coefficient.

3 Results and Discussion

3.1 *General Characteristics*

General characteristics of the subjects are shown in Table 1. The average age was 40.1 ± 2.4 years. Ten subjects were under age 40, one subject was age 40–50, and five subjects were over age 50. Five subjects did not exercise, seven subjects exercised more than two times per week, and four subjects exercised more than three times per week. Two subjects were not married and 14 subjects were married. Most subjects drank alcohol.

Table 1 General characteristics

Variables	Subject (n = 16)
Age (years)	40.1 ± 2.4[a]
≤40	10 (62.5)[b]
≤50	1 (6.3)
>50	5 (31.2)
Amount of exercise per week	
None	5 (31.3)
≥2 times/week	7 (43.7)
≤3 times/week	4 (25.0)
Marital status	
Married	14 (87.5)
Not married	2 (12.5)
Alcohols drinking	
Yes	1 (6.3)
No	15 (93.7)

[a]Means ± SE

[b]N (%)

Table 2 Body composition

Variables	Subject (n = 16)
Height (cm)	158.2 ± 1.9[a]
Weight (kg)	67.3 ± 2.4
Percent body fat (%)	23.4 ± 1.2
BMI (kg/m^2)	26.8 ± 0.8
Group 1 (18.5–24.9)	5 (31.3)[b]
Group 2 (25.0–29.9)	7 (43.7)
Group 3 (≥30)	4 (25.0)

[a]Means ± SE
[b]N (%)

Table 3 Blood lipid profiles

Variables	Subject (n = 16)
Total-cholesterol (mg/dl)	178.0 ± 31.8[a]
Triglyceride (mg/dl)	191.5 ± 112.8
HDL-cholesterol (mg/dl)	45.6 ± 9.3
LDL-cholesterol (mg/dl)	96.8 ± 23.6

[a]Means ± SE

3.2 *Body Composition*

The average height, weight, BMI, and percent body fat were 158.2 cm, 67.3 kg, 26.8 kg/m^2, and 23.3 %, respectively (Table 2). Subjects were divided into three groups according to BMI. Group 1 included five subjects, Group 2 included seven subjects, and Group 3 included four subjects. The average total cholesterol, triglyceride, HDL-cholesterol, and LDL-cholesterol were 178.0 mg/dl, 91.5 mg/dl, 45.6 mg/dl, and 96.8 mg/dl, respectively (Table 3).

3.3 *Dietary Taurine and Major Nutrient Intake*

Average dietary taurine and major nutrient intake of the groups are shown in Table 4. Average dietary taurine intake of the groups was 114.8 (Group 1), 184.3 (Group 2), and 167.6 mg/day (Group 3). The average energy intake in groups was 2,032.1 (Group 1), 2,082.6 (Group 2), and 1,842.0 kcal/day (Group 3). The average carbohydrate intake in groups was 292.4 (Group 1), 299.5 (Group 2), and 253.8 g/day (Group 3). The average total protein intake of the groups was 76.0 (Group 1), 61.2 (Group 2), and 63.5 g/day (Group 3). The average total fat intake of the groups was 63.6 (Group 1), 84.3 (Group 2), and 70.0 g/day (Group 3). No significant differences were observed for dietary taurine and the major nutrient intake among the three groups.

Table 4 Dietary taurine and major nutrient intake

Variables	Group 1	Group 2	Group 3
Taurine (mg/day)	114.8 ± 60.7[a]	184.3 ± 35.0	167.6 ± 78.0[ns]
Energy (kcal/day)	2,032.1 ± 71.6	2,082.6 ± 117.2	1,842.0 ± 39.9
Carbohydrate (g/day)	292.4 ± 12.7	299.5 ± 16.9	253.8 ± 27.5
Total protein (g/day)	76.0 ± 6.1	84.3 ± 8.9	70.0 ± 5.7
Total fat (g/day)	63.6 ± 2.2	61.2 ± 4.5	63.5 ± 12.5

[a]Means ± SE
[ns]Not significant among the three groups by one-way analysis of variance, Group 1; Normal and overweight (BMI; 18.5–24.9), Group 2; ObesityI (BMI; 25.0–29.9), Group 3; ObesityII (BMI; over 30)

Table 5 Quality of sleep score by BMI groups

	Quality of sleep score		
Item of quality of sleep	Group 1 (n = 5)	Group 2 (n = 7)	Group 3 (n = 4)
Take a long time to fall asleep	2.8 ± 0.8[a]	2.7 ± 0.7	2.5 ± 0.5[ns]
Usually do fitful sleep	3.0 ± 0.7	2.7 ± 0.7	2.3 ± 0.4
Difficult to fall asleep again during fitful sleep	3.0 ± 0.7	3.0 ± 0.5	2.8 ± 0.4
Move a lot during sleep	2.8 ± 0.8	2.7 ± 0.7	2.3 ± 0.4
Not deeply sleep	3.0 ± 0.7	3.3 ± 0.5	2.8 ± 0.4
Have a trouble sleep disturbing	3.0 ± 0.7	3.3 ± 0.7	2.8 ± 0.4
Not enough sleep because of dream	3.0 ± 0.7	3.3 ± 0.7	2.8 ± 0.4
Affect daily life that not enough sleep	3.0 ± 0.7	3.1 ± 1.0	3.0 ± 0.7
Concern that not sleep	3.2 ± 0.8	3.4 ± 0.7	3.0 ± 0.7
Too tired that wake up	2.4 ± 1.1	2.6 ± 1.0	2.8 ± 1.1
Not sleep at night	3.2 ± 1.3	3.3 ± 1.0	2.8 ± 1.1
Not enough sleep	2.8 ± 0.8	2.9 ± 0.8	3.0 ± 0.7
Sleeping more nap	2.8 ± 1.0	3.3 ± 0.9	3.0 ± 1.2
Waking up in the morning, not getting out of bed	2.0 ± 0.7	2.9 ± 1.1	3.0 ± 0.7
Continuously sleepy in the morning	2.2 ± 0.8	2.4 ± 0.7	3.3 ± 0.8

[a]Means ± SE
[ns]Not significant among the three groups by one-way analysis of variance Group 1; Normal and overweight (BMI; 18.5–24.9), Group 2; ObesityI (BMI; 25.0–29.9), Group 3; ObesityII (BMI; over 30)

3.4 *Quality of Sleep Score*

Sleep scores of the subjects are shown in Table 5. No significant difference in average quality of sleep score was observed among the groups. No significant difference in quality of sleep scores was observed among the groups. It was previously reported that an increase of BMI was associated with short sleep duration (Kohatsu 2006).

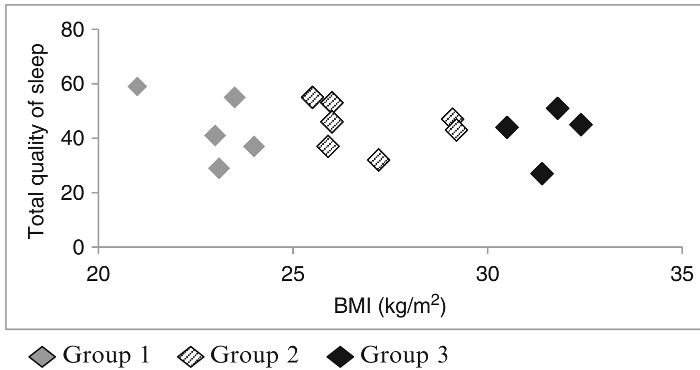

Fig. 1 Total quality of sleep and BMI, Group 1; Normal and overweight (BMI; 18.5–24.9), Group 2; ObesityI (BMI; 25.0–29.9), Group 3; ObesityII (BMI; over 30)

Table 6 Correlation between BMI and blood lipid profiles

	BMI
Total cholesterol	0.446[a]
Triglyceride	0.698**
HDL-cholesterol	–0.028
LDL-cholesterol	0.522*

BMI body mass index
[a]Pearson's correlation coefficient
*P<0.05; **P<0.01

3.5 *Total Quality of Sleep and BMI*

Quality of sleep and BMI are shown in Fig. 1. A higher BMI tended to reduce total quality of sleep. It was previously reported that poor sleep quality in adolescents may play a significant role in obesity (Gupta et al. 2002).

3.6 *Correlation Between BMI and Blood Lipid Profiles*

A correlation between blood lipid profiles and BMI of subjects is shown in Table 6 and the distribution between BMI and blood lipid profiles is shown in Fig. 2. A positive correlation was observed between BMI and blood total-cholesterol ($p<0.05$), triglyceride (0.01), and LDL-cholesterol level ($p<0.05$).

A previous study reported that obesity is a risk factor for hyper-cholesterolemia (Friedland et al. 2002).

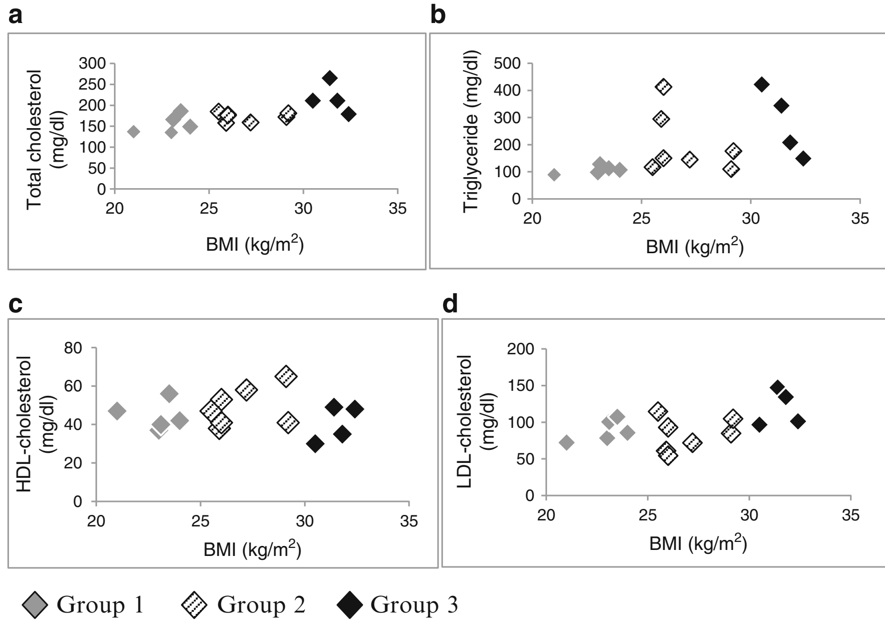

Fig. 2 Distribution between BMI and blood lipid profiles. (**a**) Between total cholesterol and BMI, (**b**) Between triglyceride and BMI. Higher BMI showed significant correlation with higher levels of blood triglyceride. (**c**) Between HDL-cholesterol and BMI. (**d**) Between LDL-cholesterol and BMI

3.7 Correlation Between Dietary Taurine Intake and Quality of Sleep

A positive correlation was observed between dietary taurine intake and the quality of sleep score (Table 7). In particular, positive correlations were observed between dietary taurine intake and sleep scores of questions including 'take a long time to fall asleep ($p<0.05$)', 'difficult to fall asleep again during fitful sleep ($p<0.05$)', and 'continuously sleepy in the morning ($p<0.05$)'. In addition, positive correlations were observed between dietary taurine intake and the total quality of sleep score. However, no positive correlation was observed between the intake of other nutrients and quality of sleep (data were not shown).

4 Conclusion

Our study investigated the association between dietary taurine intake, obesity, and quality of sleep in Korean women. These results indicate that a higher BMI tends to lower quality of sleep while dietary taurine intake tends to increase quality of sleep.

Table 7 Correlation between dietary taurine intake and quality of sleep

Item of quality of sleep	Dietary taurine intake
Take a long time to fall asleep	0.615[a]*
Usually do fitful sleep	0.459
Difficult to fall asleep again during fitful sleep	0.539*
Move a lot during sleep	0.431
Not deeply sleep	0.493
Have a trouble sleep disturbing	0.438
Not enough sleep because of dream	0.021
Affect daily life that not enough sleep	0.317
Concern that not sleep	0.252
Too tired that wake up	0.220
Not sleep at night	0.340
Not enough sleep	0.497
Sleeping more nap	0.409
Waking up in the morning, not getting out of bed	0.153
Continuously sleepy in the morning	0.515*
Total quality of sleep	0.566*

[a]Pearson's correlation coefficient

*$p<0.05$

Therefore, in order to reduce obesity, it is necessary to provide better quality of sleep and dietary taurine intake. .Further conduct of a large-scale study or case-control study is needed in order to clarify the relationship between dietary taurine, obesity, and quality of sleep.

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Taurine Supplementation Leads to a Disruption in Energy Homeostasis in Menopausal Obese Mice

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Abbreviations

AST	Aspartate aminotransferase
BAT	Brown adipose tissue
BW	Body weight
C	Chow diet
CHOL	Cholesterol
CT	Chow diet supplemented with taurine
EE	Energy expenditure
H and HFD	High fat diet
HT	High fat diet supplemented with taurine
pAkt	Phospho-Akt
RP	Retroperitoneal fat pad
TAU	Taurine
TG	Triglycerides
UCP-1	Uncoupling protein 1

1 Introduction

It is widely known that overweight and obesity are increasing around the world. In 2008, according to the World Health Organization (WHO), 12 % of the global population (aged 20 years or more) was obese, especially in developed countries

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(WHO 2008a). Accompanying fat accumulation in the body, the prevalence of some diseases related to obesity is also increasing in the population, such as insulin resistance and type II diabetes. Globally, around 9 % of adults aged 25 years old or more had raised blood glucose in 2008 (WHO 2008b). Beyond regular physical activities and weight loss, the pursuit for complementary strategies is necessary to effectively reduce the prevalence of such diseases.

Pre-menopausal women exhibit a lower prevalence of insulin resistance and cardiovascular diseases when compared to men of the same age and body mass index (BMI). These findings are largely attributed to the higher production of estrogen by pre-menopausal women over men (Freedman et al. 2004; Go et al. 2013). However, the estrogen-mediated health benefits are lost after menopause, as women experience more diseases related to the accumulation of abdominal fat than men of the same age and BMI (Zhu et al. 2014).

The relationship between menopause and weight gain, android fat distribution and decreased energy expenditure (EE) is well reported in the literature (Meli et al. 2004; Eshtiaghi et al. 2010). The drop of estradiol production with a concomitant elevation of circulating androgens leads to a decreased lean body mass and increased fat accumulation in the central region of the body (Griebeler et al. 2011), which causes a decline in basal metabolism (Palasuwan et al. 2011). Besides, according to recent studies, postmenopausal women are more likely to ingest larger quantities of saturated fat, as well as calories, which favors weight gain (Santos et al. 2008, 2011).

Taurine (TAU) is a semi-essential amino acid whose endogenous production is insufficient without diet supplementation (Ito et al. 2012). During the last few years, the beneficial effects of this amino acid on metabolic profile have been studied, including TAU effect on insulin resistance and energy metabolism (Tsuboyama-Kasaoka et al. 2006; Carneiro et al. 2009; Das et al., 2012). Studies have provided evidence for TAU as an important regulator of glucose homeostasis. For instance, supplementation of drinking water with TAU improves peripheral insulin sensitivity (Batista et al. 2013b). Moreover, in vitro treatment with TAU has been shown to increase glucose and insulin tolerance as well as to increase calcium uptake by the islets of Langerhans (Ribeiro et al. 2012a, b). Clinical data have also shown a reduction in insulin hypersecretion in obese men using TAU as a prophylactic treatment (Xiao et al. 2008). However, the beneficial effects of this amino acid on the metabolic profile of menopausal subjects remain unknown.

As efficient strategies are required to prevent and reduce the prevalence of obesity and its comorbidities, particularly after menopause, we aimed to assess growth parameters, whole-body energy expenditure, food intake and hypothalamic insulin-sensitivity in ovariectomized mice fed a high fat diet (HFD) supplemented with TAU.

2 Methods

2.1 Animals and Experimental Design

Experimental protocol for this study was approved by the Ethical Committee of State University of Campinas (UNICAMP) (Protocol 2999-1/2013) in agreement with the Guide for the Care and Use of Laboratory Animals (NIH, no. 85-23 revised 1996). Four-week old female C57BL/6J mice were purchased from the breeding colony at UNICAMP. Upon arrival, mice were maintained at a temperature/humidity controlled room (22 ± 1 °C) on a 12-h light/dark cycle (lights on 0600–1800 h), with free access to food and water. Up to 90 days of life, mice were fed with a standard control diet (C) containing 14 % protein, after which animals underwent bilateral ovariectomy, performed under general i.p. anesthesia, ketamine (Vetbrands, Paulínia, SP, Brazil) + xylazine (Rompum, Bayer, São Paulo, SP, Brazil) (Damy et al. 2010). Vaginal epithelium cell smear analysis was performed 1 month after surgery. The presence of leukocytes in 2 consecutive days of analysis is an indicator of constant diestrus and successful ovariectomy. One week after recovery from surgery, mice were either kept under a control diet (C group) or received HFD (H group) (35 % of saturated fat) for 14 weeks. Half of both C and H groups received drinking water supplemented with 3 % taurine (TAU) (CT or HT groups) until the end of the experiment. Diets were developed in accordance to AIN-93 guidelines for adult rodents and their nutritional composition was previously described (Reeves et al 1993; Batista et al. 2013b).

2.2 Nutritional Assessment

Body weight and food intake were measured once a week throughout the experimental period (14 weeks). At the end of 14 weeks, fasted mice were euthanized in a CO_2 chamber followed by decapitation. Blood samples were collected into heparinized tubes (5,000 IU diluted 1:1,000) and centrifuged at 1,600 rpm for 15 min; plasma was stored at −20 °C until use. Samples of liver, hypothalamus and brown adipose tissue (BAT) were stored at −80 °C. Commercial kits (Roche Diagnostics, Germany) were used according to the manufacturer's instructions for quantification of total plasma cholesterol (CHOL) and triglycerides (TG). Fresh plasma samples were used for quantification of aspartate aminotransferase (AST) and followed specifications from kit's manufacturer (Laborclin, Pinhais, Brazil). Fresh liver tissues were collected for histological analysis according to Ribeiro et al. (2012a, b).

2.3 Energy Expenditure and Locomotor Activity

During the last week of treatment, animals had their energy expenditure (EE) and spontaneous locomotor activity evaluated. To perform indirect calorimetry, mice were housed in metabolic cages for 24 h acclimation. Next, oxygen (O_2) consumption and carbon dioxide release (CO_2) were measured for additional 24 h using the Oxylet system (PanLab/Harvard Instruments, Barcelona, Spain). Respiratory quotient (RQ) and energy expenditure (EE) were later calculated using the Metabolism® software (PanLab/Harvard Instruments, Barcelona, Spain). To perform spontaneous locomotor activity, mice were initially housed in Multitake Cage LE 001 PH (PanLab/Harvard Instruments, Barcelona, Spain) for 24 h to ensure acclimation, after which spontaneous activity was recorded for additional 24 h. Data analysis was performed using the Compulse® v. 2.7.13 software.

2.4 Western Blot Analysis

For protein expression experiments, 12-h fasted mice from all groups had their hypothalamus and brown adipose tissue (BAT) removed and immediately homogenized in buffer containing 100 mmol/L Tris pH 7.5, 10 mmol/L sodium pyrophosphate, 100 mmol/L sodium fluoride, 10 mmol/L EDTA, 10 mmol/L sodium vanadate, 2 mmol/L PMSF, and 1 % Triton X-100. The tissue extracts were centrifuged at 12,000 rpm at 4 °C for 40 min to remove insoluble material. An additional 20 min of centrifugation was necessary for BAT. The protein concentration in the supernatants was assayed using the Bradford dye method (Bradford 1976), using bovine serum albumin (BSA) as a standard (Bio-Agency Lab., São Paulo, SP, Brazil). For SDS gel electrophoresis and Western Blot analysis, supernatants were treated with Laemmli sample buffer containing dithiothreitol. After sample boiling for 5 min, the proteins were separated by electrophoresis (50 μg protein/lane, 10 % acrylamide). Following electrophoresis, proteins were transferred to nitrocellulose membranes. Membranes were treated with a blocking buffer for 2 h (5 % nonfat dried milk, 10 mmol/L Tris, 150 mmol/L NaCl and 0.02 % Tween 20) and then, incubated overnight with a polyclonal antibody against p-Akt Ser (1:1,000, cat. Sc-7985R, Santa Cruz Biotechnology), total Akt (1:1,000, cat. Sc-8312, Santa Cruz Biotechnology) and UCP-1 (1:500, cat. Sc-6529, Santa Cruz Biotechnology). Next, secondary anti-rabbit antibody was incubated at 1:10,000 dilution at room temperature for 1 h. Imaging and densitometry were performed using the Image Quant LAS 4000 Mini (GE® Healthcare Bio-Sciences, Uppsala, Sweden) imaging system and Image J 1.47 (NIH, USA) processing program. The values obtained from p-Akt were normalized by total Akt expression, as previously described (Batista et al. 2012; Ribeiro et al. 2012a, b). Glyceraldehyde 3-phosphate dehydrogenase protein (GAPDH; 1:1,000, cat. sc-25778, Santa Cruz Biotechnologies, CA, USA) was used as loading control.

2.5 Statistical Analysis

Data are presented as means ± SEM. Differences between groups were determined either by two-way ANOVA, followed by Holm-Sidak posttests, or Student's t test. Significance was considered as $P < 0.05$. Statistics were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA) and SigmaStat for Windows version 3.5 (Dundas Software, Germany).

3 Results

3.1 Mice Features

Figure 1 shows an increase in body weight (BW) of H and HT groups compared with C and CT mice between the 1st and 14th week of treatment (Fig. 1a). BW expressed by the area under the curve (AUC) during experimental period was also higher in H and HT mice (C vs. H, $P = < 0.001$; H vs. HT, $P = 0.02$; Fig. 1b). At the end of the 14th week of experiment, BW and retroperitoneal fat pads (RP) were higher in H and HT mice compared with their controls (Table 1).

Fasting plasma cholesterol (CHOL) and aspartate aminotransferase (AST) were higher in H and HT mice when compared to C and CT (Fig. 2a, b), whereas plasma triglycerides (TG) were higher only in the HT group when compared with C and CT mice (Fig. 2c). HFD supplementation with TAU (HT group) led to increased CHOL and AST levels. In a separate analysis between H and HT groups, TAU supplementation seems to increase TG values ($P = 0.07$). In addition, a greater fat accumulation is present in the liver of H and HT mice in relation to their controls, indicating hepatic steatosis (Fig. 2d).

3.2 Energy Metabolism

At the last week of the experimental period (14th week), mice energy metabolism was assessed through indirect calorimetry and spontaneous locomotor activity. Brown adipose tissue (BAT) UCP-1 protein expression was also analyzed in order to evaluate energy expenditure. Figure 3a shows that during the dark phase of the daily cycle, when animals are more active, there was no difference in energy expenditure (EE) for H and HT mice in comparison to their controls. In contrast, H and HT mice had a lower respiratory quotient (RQ) in the dark phase than C and CT animals (Fig. 3c). C and CT mice were able to switch their substrate oxidation during light and dark phases whereas H and HT mice did not exhibit this capacity, indicating that obese mice are metabolically inflexible.

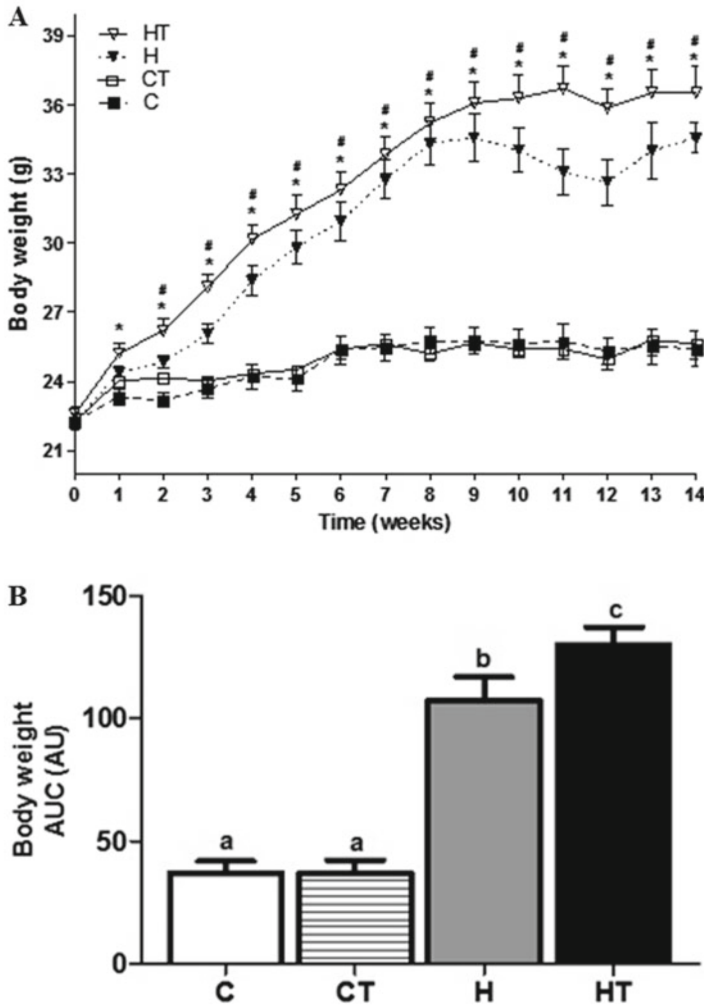

Fig. 1 (**a**) Body weight during the experimental period was measured weekly in mice fed with standard control (C) or high fat (H) diets and with or without taurine (TAU) supplementation (CT or HT groups). (**b**) Mean ± SEM of AUC of body weight registered during 14 weeks of diet and T treatments. *Symbol* (*) means difference between C and H groups and (#) means difference between H and HT mice. *Different letters over the bars* represent significant differences between groups ($P < 0.05$)

Table 1 Growth parameters (BW and retroperitoneal fat pad weight in C, CT, H and HT mice)

	C	CT	H	HT
BW (g)	21.1 ± 0.4^a	24.2 ± 0.3^a	33.4 ± 0.9^b	36.3 ± 0.8^c
Retroperitoneal fat pad (% BW)	0.5 ± 0.04^a	0.5 ± 0.06^a	1.7 ± 0.21^b	2.1 ± 0.32^c

Data are presented in means ± SEM values. *BW* body weight. Different letters indicate $P < 0.05$ between groups

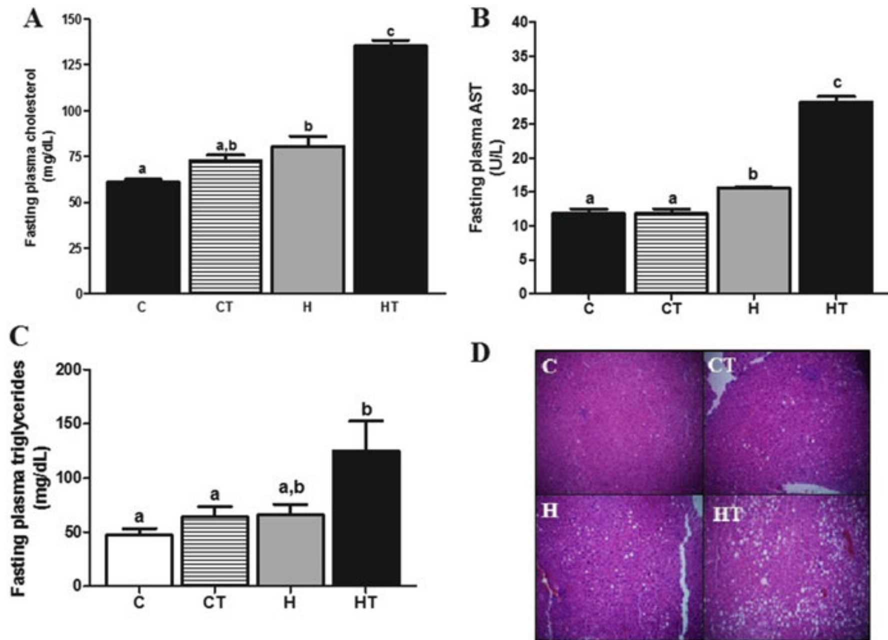

Fig. 2 (**a**) Fasting plasma cholesterol values at the end of the experimental period in mice fed with standard control (C) or high fat (H) diets supplemented or not with taurine (CT and HT groups). (**b**) Fasting plasma aspartate aminotransferase (AST) for C, CT, H and HT groups. (**c**) Fasting plasma triglycerides (TG) for C, CT, H and HT mice. The *bars* represent means ± SEM values. *Different letters over the bars* mean significant differences between groups ($P < 0.05$). (**d**) Histological sections of liver stained with hematoxylin for C, CT, H and HT mice

Mice fed a HFD exhibited lower spontaneous locomotor activity during the dark phase when compared with their controls (Fig. 4a, b). UCP-1 protein expression from brown adipose tissue (BAT) was significantly higher in H mice and this increase could be inverted by HFD supplementation with TAU (Fig. 4c).

3.3 *Food Intake and Hypothalamic Insulin Signaling*

Food intake was measured throughout the experimental period (14 weeks). Figure 5a, b shows food intake of C, CT, H and HT groups. Mice fed a HFD had lower food intake when compared with its control (Fig. 5a). Despite lower food intake, the calorie content ingested in a week by H mice was comparable to C and CT controls, consistent with the higher calorie value of a HFD (Fig. 5b). HT mice exhibited a lower food intake in grams when compared with H, with a consequent decreased food intake given in calories ($P = 0.03$ and $P = 0.06$, respectively; Fig. 5a, b).

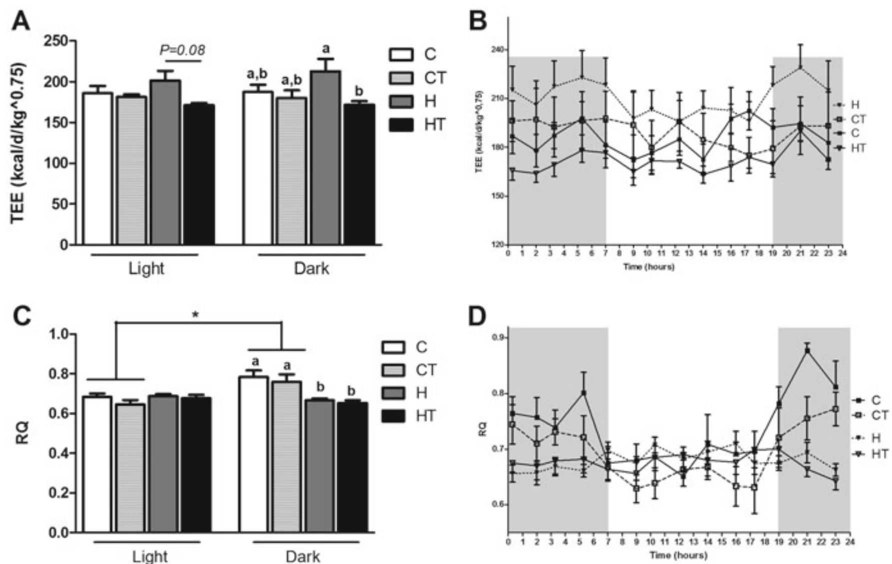

Fig. 3 (**a**) Energy expenditure (EE) measured by indirect calorimetry for C, CT, H and HT groups. (**b**) Representation of EE for all groups during light and dark phases. (**c**) Respiratory quotient (RQ) calculated as (Expired CO_2 volume/inspired O_2 volume) for all groups. (**d**) Representation of RQ for all groups during light and dark phases. *Bars* represent means ± SEM values. *Different letters over the bars* denote significant differences between groups ($P < 0.05$)

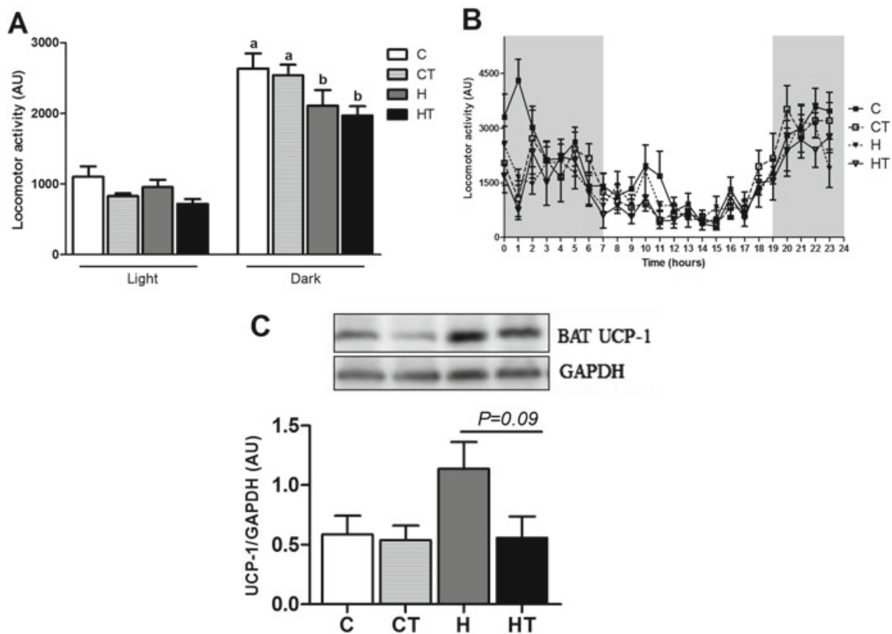

Fig. 4 (**a**) Spontaneous locomotor activity for all groups. (**b**) Representation of locomotor activity for all groups during light and dark phases. (**c**) Values of UCP-1 protein expression evaluated through western blot. *Bars* represent means ± SEM values. *Different letters over the bars* denote significant differences between groups ($P < 0.05$)

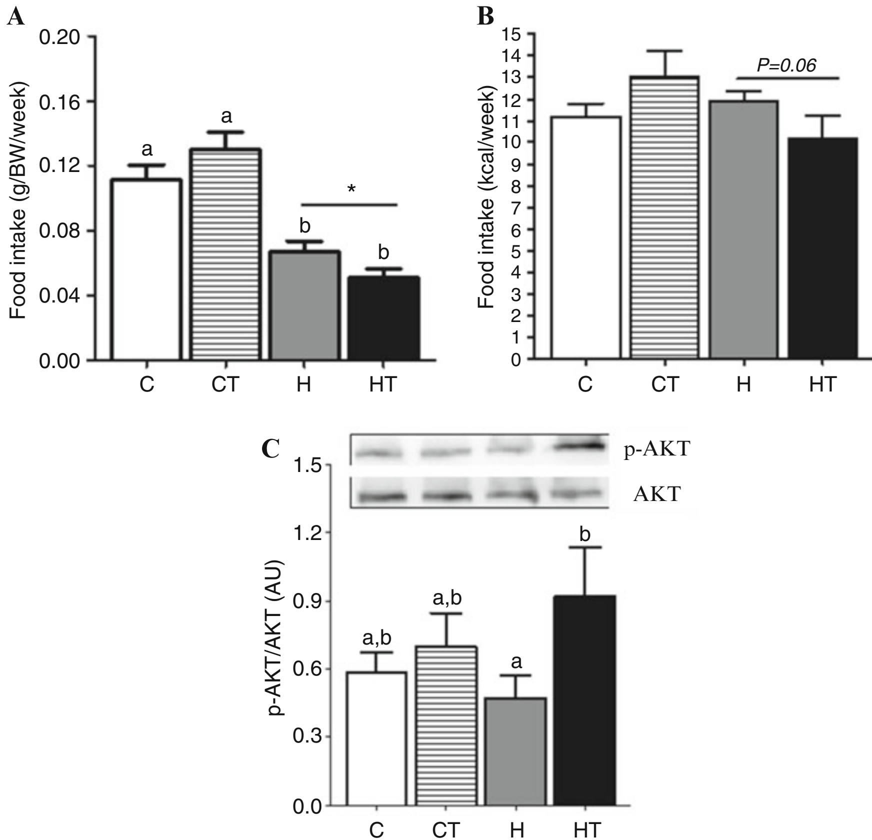

Fig. 5 (**a**) Food intake was measured weekly during the experimental period for C, CT, H and HT mice, in grams/body weight/week. (**b**) Food intake in grams/week was converted into calories/week for each group multiplying the value by 3.88 kcal/g for standard control (C) and 5.44 kcal/g for high fat diet (HFD) (Batista et al. 2013b). *Different letters over the bars* denote significant differences between groups ($P < 0.05$). (**c**) Values of pAkt/Akt protein phosphorylation. The *bars* represent means ± SEM values. *Different letters over the bars* denote significant differences ($P < 0.05$). (*) means $P < 0.05$ using t-Student

To further investigate the insulin-sensitivity pathway and its relation to food intake, a ratio between the densitometry of phosphorylation (pAkt) and total Akt content was performed (Ribeiro et al. 2012a, b). Figure 5c shows no difference in Akt phosphorylation between C, CT and H groups. However, HFD association with-TAU caused an increase in Akt phosphorylation in comparison to the H group ($P < 0.05$; Fig. 4c).

4 Discussion

In agreement with our previous studies using male mice, HFD intake by female, menopaused-mice increased body weight and fat pads for both the H and HT groups, leading to obesity (Batista et al. 2013b; Camargo et al. 2013; Vettorazzi et al. 2014). In addition, HFD intake also increased mice plasma CHOL, AST and led to diminished spontaneous locomotor activity. According to the American College of Obstetrics and Gynecologists (2005) and other studies (Guthrie and Dinnerstein 2003; Dubnov-Raz et al. 2007), there is an increased prevalence of obesity after menopause due to decreased body lean mass, lower EE (Poehlman et al. 1998; Luhrmann et al. 2009) and redistribution of body fat from gynoid to android type, all of which favor lipogenesis (D'Eon et al. 2005; Paz et al. 2006). Weight gain during the post-menopause phase of life is further reinforced by erroneous dietary intake, especially due to saturated fat, as reported by Santos et al. (2008, 2011).

In our study, when TAU was administered with the control diet (CT group), no difference was observed in relation to the C group in the parameters described above. However, TAU induced a significant increase in body weight of HFD-fed mice that surpassed the weight gain observed for mice under HFD only. These effects were observed early during treatment and were maintained throughout the duration of the studies. In addition to body weight, TAU exacerbated the levels of plasma CHOL, AST, TG, and liver steatosis. Recent studies have demonstrated that TAU addition to the diet or drinking water reduces blood lipid levels both in rodent models and humans (Fukuda et al. 2011; Nardelli et al. 2011). Tastesen et al. (2014) showed that seafood—which contains large quantities of TAU-intake by male mice resulted in decreased lipid levels in the plasma. Our observed increase in plasma levels of mice fed with HFD and treated with TAU suggests that TAU mediated an alteration in energy metabolism, and this merits further investigation. Also, it is already known that estrogen is an important regulator of energy and glucose metabolism (D'Eon et al. 2005; Luhrmann et al. 2009), and the disruption of the estrogen signaling pathway in OVX mice appears to prevent the beneficial effects of TAU supplementation.

Several studies suggest that TAU could mediate a reduction in obesity (Cappelli et al. 2014; Murakami et al. 2000; Nardelli et al. 2011). For instance, increased TAU concentration in the plasma following its intake was correlated with prevention of the development of obesity (Tsuboyama-Kasaoka et al. 2006). Nardelli et al. (2011) showed that TAU treatment leads to a reduction in body adiposity, without affecting body weight in obese rats. Moreover, experimental models of obesity exhibit decreased TAU biosynthesis by adipose tissue, as well as lower levels of TAU in the plasma (Tsuboyama-Kasaoka et al. 2006). Diet and genetic-obesity development, as well as liver damage and hypercholesterolemia, can be prevented with TAU supplementation (Tsuboyama-Kasaoka et al. 2006). However, Murakami et al. (2000) showed no reduction in body weight of HFD-fed-mice treated with TAU. We suggest the exacerbated increase in weight gain, adipose fat pads, and lipid parameters of HT mice in this study in comparison with the H group could be explained at least in part by the decrease in EE from HT mice in relation to the H group.

After menopause, resting EE decreases 2–10 % per decade (FEBRASGO 1995), which can occur concurrently with a reduction in EE induced by exercise (Luhrmann et al. 2009). Estrogen deficiency associated with weight gain is also related to metabolic inflexibility (Berk et al. 2009). Despite lower spontaneous locomotor activity when subjected to HFD, there was no significant difference in EE between mice fed the control diet or HFD. Interestingly, only mice fed a control diet were able to shift their substrate oxidation between light and dark cycles. In agreement with a previous report (Berk et al. 2009), our HF mice exhibited light–dark metabolic inflexibility, which can be attributed to their lower locomotor activity.

TAU supplementation increases adipocyte mRNA levels of transcription factors and cofactors involved in EE, as well as mRNA levels of fatty acid β-oxidation enzymes and mitochondrial respiration, components that favors greater EE (Tsuboyama-Kasaoka et al. 2006). In our model of TAU supplementation, mice fed a HFD showed lower EE in comparison to the H group. When we investigated UCP-1 protein expression from BAT, a decrease in protein levels was observed for HT mice when compared with the H group ($P=0.09$, Fig. 3g). This decrease can be attributed to the lower EE in HT mice. Therefore, we believe that the association of TAU with HFD in menopausal mice model causes a modulation in energy expenditure possibly because of TAU modulation of UCP-1 from BAT, which would at least partially explain the higher body weight and retroperitoneal fat depots observed in HT mice (Table 1).

In the last part of our study, we investigated food intake by our animals and its effect on hypothalamic insulin sensitivity. Mice fed HFD had a lower food intake compared with control diet-fed mice possibly because of lower palatability of HFD over time. Although all groups ingested almost the same quantity of calories, HT group exhibited higher body weight and fat depots, pointing out TAU as a modulator of energy metabolism. When TAU was associated with HFD, mice presented a lower food intake as previously described (Batista et al. 2013b; Cappelli et al. 2014; Camargo et al. 2013), possibly because its anorexigenic effects. Previous studies demonstrated that TAU is able to improve the effects of insulin in the hypothalamus, which promotes a reduction in the expression of the orexigenic neuropeptide Y without altering the expression of the anorexigenic neuropeptide POMC (Solon et al. 2012). Consistent with these findings, we observed improved hypothalamic insulin sensitivity given by the increased activation of Akt in animals supplemented with TAU. Camargo et al. (2013) also demonstrated increased p-Akt/Akt activation in obese animals supplemented with TAU in the drinking water when compared to untreated group.

5 Conclusion

TAU supplementation in menopausal mice fed a HFD developed obesity, increased adiposity and hypercholesterolemia. Surprisingly, TAU supplementation enhanced these deleterious effects through modulation of central and peripheral pathways leading to lower EE and food intake. These latter effects were related to increased hypothalamic insulin-sensitivity.

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Effects of Paternal Hypothalamic Obesity and Taurine Supplementation on Adiposity and Vascular Reactivity in Rat Offspring

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Abbreviations

Ach	Acetylcholine
AUC	Area under curve
BW	Body weight
CHOL	Cholesterol
CTAU	CTL supplemented with Tau
CTL	Control
ipGTT	Intraperitoneal glucose tolerance test
MSG	Monosodium glutamate
MTAU	MSG supplemented with Tau
NO	Nitric oxide
Phe	Phenylephrine
RIA	Radioimmunoassay
Tau	Taurine
TG	Triglycerides

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1 Introduction

Obesity is characterized by excessive fat accumulation, due to an imbalance between calories consumed and expended. This syndrome is associated with an increased risk of type 2 diabetes and cardiovascular diseases (Meigs 2010). Genetic, neuroendocrine, metabolic, behavioral and environmental factors contribute to the manifestation and maintenance of obesity (Soubry et al. 2013; Sominsky and Spencer 2014; Chavey et al. 2014).

Several reports have demonstrated that the intrauterine milieu and changes in maternal metabolism may program the metabolic phenotype of the offspring through physiological and/or epigenetic mechanisms (Cherif et al. 1996; Kalbe et al. 2005; Catalano et al. 2009; Chavey et al. 2014). Maternal diabetes and/or obesity were also associated with cardiovascular dysfunction in adult offspring and increased mortality from cardiovascular events (Dabelea 2007; Reynolds et al. 2013). However, while paternal metabolic conditions are often not considered, paternal metabolism can alter DNA and histone methylation and acetylation profiles, leading to alterations in gene expression. These epigenetic modifications are propagated through the gametes to the offspring and may contribute to the development of metabolic dysfunctions and its associated comorbidities in later life (Ng et al. 2010; Soubry et al. 2013; Fullston et al. 2013).

Neonatal administration of monosodium glutamate (MSG) in rodents causes lesions in different hypothalamic areas, resulting in metabolic and neuroendocrine changes that lead to obesity (Olney 1969). MSG obese rodents show massive fat accumulation (Balbo et al. 2000), hyperleptinemia (Kim et al. 2005), hyperinsulinemia, insulin resistance and hypertriglyceridemia (Hirata et al. 1997; Nardelli et al. 2011). In addition, MSG hypothalamic obesity also causes vascular dysfunction with lower nitric oxide (NO) bioavailability and increased reactive oxygen species production in vascular beds (Lobato et al. 2011).

The amino acid taurine (Tau) is present in high levels in mammalian tissues and plays a role in the regulation of fat metabolism, in body glucose control and in cardiovascular function (Ribeiro et al. 2009; Xu et al. 2008; Nardelli et al. 2011; Abebe and Mozaffari 2011). In the former process, Tau demonstrates antihypertensive actions (Abebe and Mozaffari 2011), vasorelaxation properties (Ristori and Verdetti 1991; Niu et al. 2008; Abebe and Mozaffari 2011) and retards the initiation and progression of atherosclerosis (Murakami 2014). Here, we analyzed whether paternal MSG hypothalamic obesity may influence adiposity, glucose tolerance and vascular reactivity in later life in offspring. We also verified whether Tau supplementation given to fathers modulates these effects.

2 Methods

2.1 MSG Treatment, Tau Supplementation and Offspring Groups

All protocols were approved by the local Animal Care and Use Committee (CEUA *Campus UFRJ-Macaé*, license n°.: MACAE01). Male *Wistar* rats received subcutaneous injections of MSG [4 g/kg body weight (BW); MSG group] or hyperosmotic saline solution [1.25 g/kg BW; control (CTL) group] during the first 5 days of life (Balbo et al. 2000). At weaning (21 days), the rats were distributed into the following groups: CTL, MSG, and CTL and MSG supplemented with 2.5 % Tau in their drinking water (CTAU and MTAU, respectively). At 90 days of age, all male rat groups were mated with 90-day-old female CTL rats to obtain offspring that were designed according to their fathers' treatment as: CTL, CTAU, MSG and MTAU. During mating, one male and three females were housed together for 8 consecutive days. Litter sizes were standardized to eight pups at postnatal day 1 to control nutrition during the lactation period.

All rat groups were maintained on a 12 h light/dark cycle (lights on 07:00–19:00 h), controlled temperature (21 ± 2 °C) and allowed free access to food and water. All experimental procedures listed below were developed in the male founders at 90 days of age. For female and male offspring groups, the experimental protocols were evaluated at 150 days of age. During the entire experimental period, offspring groups only consumed standard food and water.

2.2 General Nutrition Parameters

Body weight and food intake were measured once a week during the entire experimental period in all offspring groups. At the end of the experimental period, all rats were weighed and nasoanal lengths were measured to calculate the Lee index [BW (g)$^{1/3}$/nasoanal length (cm) × 1,000] (Bernardis and Patterson 1968). Subsequently, rats were euthanized by cervical dislocation and perigonadal and retroperitoneal fat pads were collected and weighed. Blood samples were collected from the tip of the tail and the plasma was used for insulin measurement by radioimmunoassay (Ribeiro et al. 2010), and total cholesterol (CHOL) and triglyceride (TG) levels using standard commercial kits, according to the manufacturer's instructions (Boehringer Mannhein®, Germany; Merck®, Germany and Wako®, Germany, respectively). Plasma glucose was measured using a glucose analyzer (Accu-Chek Performa, Roche Diagnostic, USA).

2.3 Intraperitoneal Glucose Tolerance Test (ipGTT)

After a 12 h of fasting, blood samples were collected from the tip of tail from all rats in the offspring groups to measure glycemia using a glucose analyzer (Accu-Chek Performa, Roche Diagnostic, USA), before (0 min) and at 15, 30, 60 and 120 min after an i.p. injection of glucose (2 g/kg BW).

2.4 Vascular Reactivity in Thoracic Aorta

At 150 days of age, male offspring were euthanized by cervical dislocation and the thoracic aorta was immediately dissected. Adherent fat and connective tissues were carefully removed, and the aorta was cut into rings of 2–3 mm in length. The aortic rings were placed in a 10 mL vertical chamber containing Krebs Henseleit solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$ and 11 mM glucose) maintained at 37 °C, pH 7.4, and continuously gassed with carbogen (95 % O_2/5 % CO_2). Isometric tension was measured for each aorta ring mounted between two hooks, one of which was attached to a force transducer (MLT0201; ADInstruments, Bella Vista, New South Wales, Australia). Signals were conditioned (Power Lab 4/30, ADInstruments, Bella Vista, New South Wales, Australia), displayed, and stored on a computer for analysis using LabChart Pro software (ADInstruments, Bella Vista, New South Wales, Australia). After a 90 min equilibration period, concentration-response curves to phenylephrine (Phe; 0.001–10 μM) or acetylcholine (Ach; 0.001–10 μM) were recorded. Relaxations were plotted as percentages of the contraction induced by 10 μM Phe (Raimundo et al. 2006).

2.5 Statistical Analysis

Data are presented as means ± SEM, with the number of determinations (n) indicated. The pEC50 ($-\log^{EC50}$) was calculated by non-linear regression analysis. Statistical analyses were carried out using two-way analysis of variance (ANOVA) followed by the Duncan's posttest ($P < 0.05$) with the Statistica® Software version 7.0 (Statsoft, Tulsa, OK, USA) and graphs were performed using GraphPad Prism® version 5.00 (GraphPad Software, San Diego, CA, USA).

3 Results

3.1 Obesity Evaluation and General Nutritional Features in Paternal Groups

Final BW and length in MSG-treated rats were lower than those of the CTL group ($P<0.05$ and $P<0.03$; Table 1). MSG treatment efficiently induced obesity, since the perigonadal and retroperitoneal fat stores were 104 and 178 % larger in the MSG group compared to the CTL rats ($P<0.0005$ and $P<0.0001$, respectively; Table 1). Tau-supplemented MSG rats exhibited a reduction of 20 % in the perigonadal fat pads, when compared with non-supplemented MSG rats ($P<0.02$). However, Tau treatment did not alter other obesity parameters such as BW and the retroperitoneal fat depots (Table 1). In addition, despite no difference in glycemia at fasting and in a fed state between MSG and CTL rats (Table 2), normoglycemia was maintained in the MSG group by hyperinsulinemia under both conditions ($P<0.02$ and $P<0.005$). MSG rats also showed hypertriglyceridemia under these nutritional conditions ($P<0.004$), but presented normal plasma CHOL levels (Table 2). Tau supplementation normalized TG plasma levels in the fed state, without altering the other plasma biochemical parameters (Table 2).

3.2 Obesity Evaluation and General Nutritional Features in Offspring Groups

Body weight in female and male offspring groups was measured weekly, as illustrated in Fig. 1a, c, respectively. No differences between MSG and CTL offspring were observed in total BW, expressed by the area under the growth curve (AUC), as registered during the experimental period (Fig. 1b, d). Total food consumption was also similar in female and male MSG offspring (2,478 ± 16 and 2,742 ± 71 g weeks^{-1}) and in CTL offspring (2,599 ± 64 and 2,924 ± 97 g weeks^{-1}, respectively). At 150 days of age, final BW and length, and Lee index did not differ between female and male MSG and CTL offspring (Table 3).

Table 1 Obesity parameters evaluated in 90-day-old male CTL, CTAU, MSG and MTAU rats

	CTL	CTAU	MSG	MTAU
BW (g)	371 ± 13^{a}	384 ± 12^{a}	298 ± 37^{b}	307 ± 19^{b}
Nasoanal length (cm)	24 ± 0.7^{a}	24 ± 0.3^{a}	22 ± 0.6^{b}	22 ± 0.4^{b}
Lee index	300 ± 7	300 ± 1	313 ± 3	303 ± 5
Perigonadal fat pad (mg/g BW)	15.4 ± 0.6^{a}	14.3 ± 0.9^{a}	31.5 ± 2.1^{b}	25.1 ± 1.6^{c}
Retroperitoneal fat pad (mg/g BW)	12.1 ± 1.4^{a}	12.8 ± 1.4^{a}	33.7 ± 1.7^{b}	31.1 ± 1.7^{b}

Data are means ± SEM (n = 3–4 rats). Different letters represent significant differences ($P<0.05$)

Table 2 Plasma biochemical parameters in 12 h-fasted and fed 90-day-old male CTL, CTAU, MSG and MTAU rats

		CTL	CTAU	MSG	MTAU
Glycemia (mg/dL)	Fasting	105 ± 3	113 ± 1	97 ± 4	95 ± 3
	Fed	130 ± 2	136 ± 5	145 ± 10	121 ± 5
Insulinemia (ng/mL)	Fasting	0.52 ± 0.1[a]	0.75 ± 0.1[a,b]	1.18 ± 0.3[b]	1.34 ± 0.2[b]
	Fed	1.15 ± 0.3[a]	1.68 ± 0.3[a,c]	4.61 ± 0.7[b]	3.2 ± 0.9[b,c]
TG (mg/dL)	Fasting	75 ± 6[a]	75 ± 7[a]	143 ± 21[b]	116 ± 19[b]
	Fed	97 ± 7[a]	87 ± 6[a]	158 ± 7[b]	97 ± 9[a]
CHOL (mg/dL)	Fasting	50 ± 4	60 ± 6	62 ± 5	63 ± 6
	Fed	45 ± 5	53 ± 10	53 ± 3	56 ± 6

Data are means ± SEM (n = 5–7 rats). Different letters represent significant differences (P < 0.05)

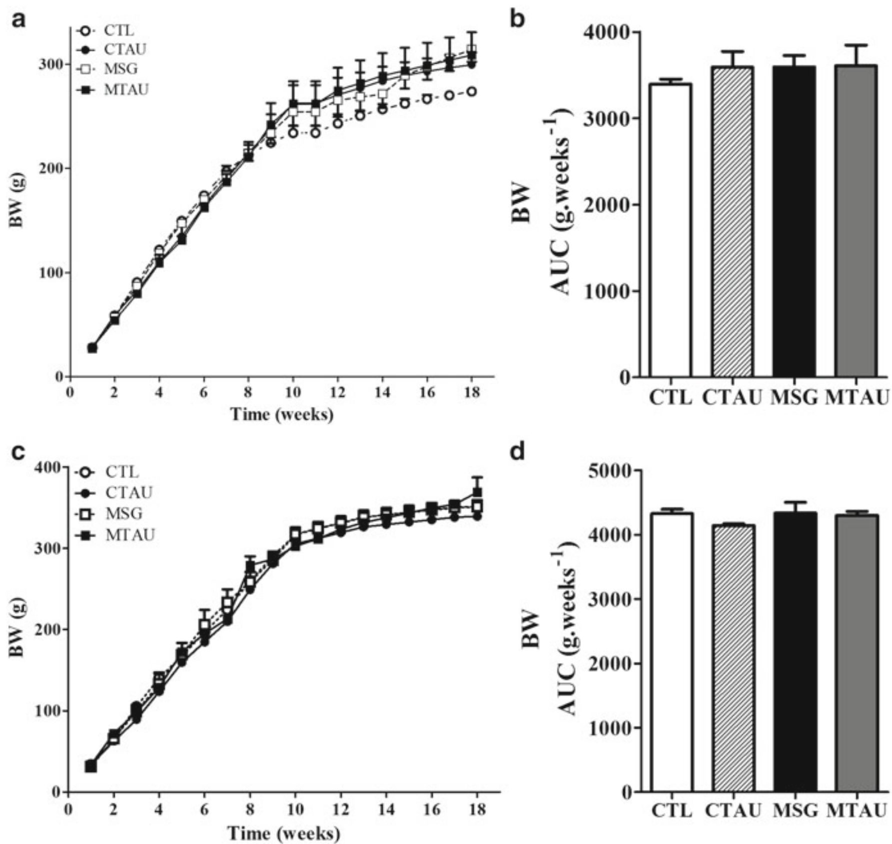

Fig. 1 Changes in body weight (BW) in female (**a**) and male (**b**) offspring from male MSG and CTL rats supplemented or not with Tau. Means ± SEM (n = 3–10) of total BW registered during 18 weeks in female (**c**) and male (**d**) offspring from male CTL, CTAU, MSG and MTAU rats

Table 3 Obesity parameters in 150-day-old female and male offspring from male CTL, CTAU, MSG and MTAU rats

		CTL	CTAU	MSG	MTAU
BW (g)	Female	232 ± 4	233 ± 4	244 ± 6	226 ± 4
	Male	382 ± 12	397 ± 6	357 ± 12	423 ± 7
Nasoanal length (cm)	Female	21 ± 0.3	22 ± 0.3	21 ± 0.2	21 ± 0.2
	Male	24 ± 0.4	25 ± 0.4	24 ± 0.3	25 ± 0.4
Lee index	Female	295 ± 4	290 ± 3	299 ± 3	295 ± 1
	Male	295 ± 2	286 ± 4	296 ± 3	299 ± 4
Perigonadal fat pad (mg/g BW)	Female	10.7 ± 2.2[a]	24.1 ± 1.8[b]	21.6 ± 1.2[b,c]	17.0 ± 2.2[c]
	Male	12.2 ± 0.8	12.7 ± 1.1	14.5 ± 1.6	13.9 ± 2.0
Retroperitoneal fat pad (mg/g BW)	Female	9.8 ± 2.1[a]	24.7 ± 1.6[b]	24.2 ± 2.8[b]	17.0 ± 2.2[b]
	Male	8.2 ± 0.5	8.1 ± 1.2	8.7 ± 0.9	9.7 ± 2.2

Data are means ± SEM (n = 3–10 rats). Different letters represent significant differences (P < 0.05)

Table 4 Biochemical parameters in 12 h-fasted 150-day-old female and male offspring from male CTL, CTAU, MSG and MTAU

		CTL	CTAU	MSG	MTAU
Glycemia (mg/dL)	Female	105 ± 2	111 ± 4	114 ± 2	104 ± 4
	Male	104 ± 3	102 ± 3	113 ± 3	110 ± 1
Insulinemia (ng/mL)	Female	0.53 ± 0.08	0.57 ± 0.15	0.65 ± 0.09	0.37 ± 0.05
	Male	1.3 ± 0.2	1.0 ± 0.2	0.9 ± 0.2	1.13 ± 0.2
TG (mg/dL)	Female	98 ± 13	101 ± 14	104 ± 18	97 ± 10
	Male	64 ± 7	84 ± 20	71 ± 14	64 ± 3
CHOL (mg/dL)	Female	85 ± 7	71 ± 7	84 ± 7	61 ± 4
	Male	96 ± 6	99 ± 11	88 ± 5	92 ± 10

Data are means ± SEM (n = 3–10 rats)

In contrast, perigonadal and retroperitoneal fat pads were 50 % and 60 % higher, respectively, in female MSG offspring, when compared with CTL offspring (P < 0.002 and P < 0.005, respectively). No difference between fat stores was observed between male MSG and CTL offspring (Table 3). In addition, both female MTAU and CTAU offspring from fathers that were supplemented with Tau presented higher perigonadal and retroperitoneal fat weights than the CTL group (P < 0.04 and P < 0.005; Table 3). In addition, plasma glucose, insulin, TG and CHOL levels were similar between offspring from MSG and CTL fathers that were supplemented or not with Tau (Table 4).

Figure 2 shows an ipGTT in 150-day-old female and male offspring from MSG and CTL fathers that were supplemented or not with Tau. Fasting glucose levels did not differ between the groups. After a glucose load, plasma glucose reached a peak at 15 min of the ipGTT in all female (Fig. 2a) and male (Fig. 2c) offspring rat groups and returns to basal glucose conditions after 120 min. No alterations in glucose

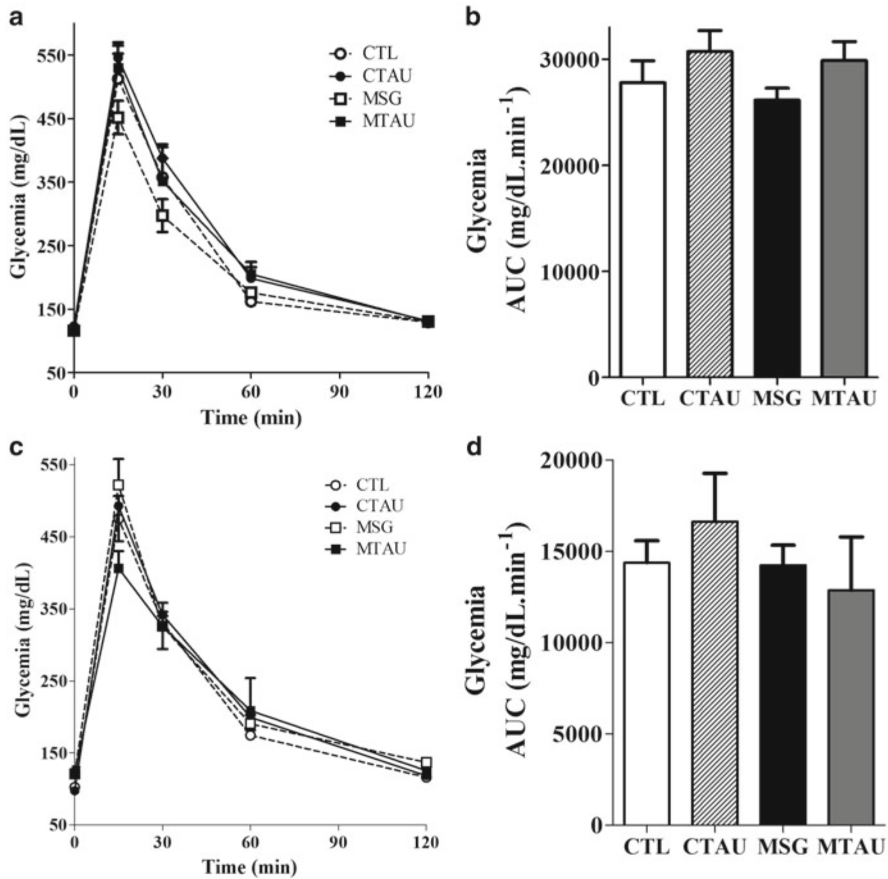

Fig. 2 Plasma glucose concentrations during an ipGTT in 150-day-old female (**a**) and male (**b**) offspring from male MSG and CTL rats supplemented or not with Tau. The figures show blood glucose levels before and after an i.p. injection of glucose (2 g/kg BW). Means ± SEM (n = 3–10) of total glycemia during ipGTT in female (**c**) and male (**d**) offspring from male CTL, CTAU, MSG and MTAU rats

levels during ipGTT or total glycemia expressed by the area under glucose curves (AUC) were observed between the groups (Fig. 2b, d).

3.3 Vascular Reactivity in Thoracic Aorta from Male Offspring

Figure 3a shows that the maximal vasoconstrictor response to Phe was similar in aortas from MSG (1.8 ± 0.1 g) and CTL male offspring (1.7 ± 0.1 g), and Tau supplementation did not alter this parameter in MTAU and CTAU offspring (1.7 ± 0.2 and

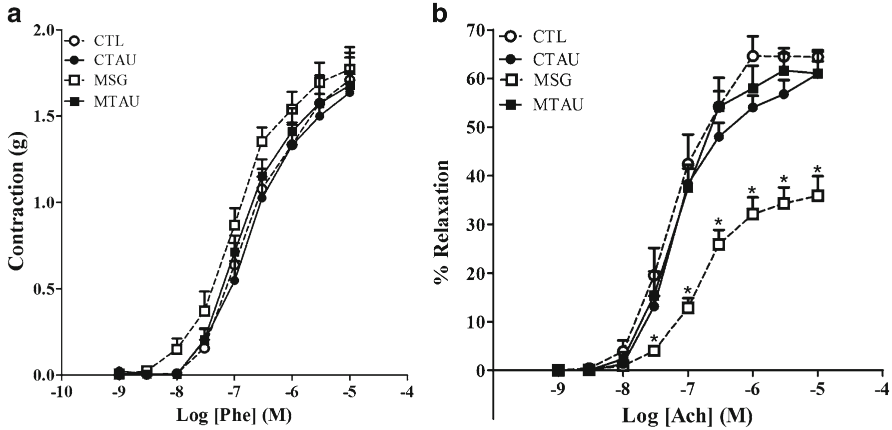

Fig. 3 Dose-response curves for Phe (**a**) and Ach (**b**) in isolated rat thoracic aortas from 150-day-old male offspring from CTL, CTAU, MSG and MTAU rats. Data are means ± SEM (n = 7–14 independent experiments). *MSG offspring is different from CTL under the same [Ach] evaluated ($P < 0.05$)

1.6 ± 0.1 g, respectively). Also, the potency of Phe was not significantly altered in aortas from MSG (pEC_{50}, MSG 7.1 ± 0.1 and CTL 6.7 ± 0.1), and after Tau supplementation (pEC_{50}, MTAU 6.9 ± 0.1 and CTAU 6.7 ± 0.1).

The dose-response relaxation curves in response to Ach (0.001–10 μM) are presented in Fig. 3b. Ach-induced maximum relaxation was significantly reduced in aortas from MSG male offspring (36 ± 4 %) in comparison with CTL aortas (64 ± 1 %; $P < 0.03$), although pEC50 was not significantly altered (pEC_{50}, MSG 4.4 ± 0.3 vs. CTL 6.4 ± 0.2; Fig. 3b). Tau supplementation in MSG fathers prevented endothelium dysfunction in their offspring, since MTAU descendants showed similar maximal relaxation (61 ± 4 %) and pEC_{50} (6.1 ± 0.2) in response to Ach as observed in CTL offspring (Fig. 3b).

Vascular reactivity of aortas from the female offspring did not differ between the groups (data not shown).

4 Discussion

Our data show that Tau supplementation prevented fat deposition and hypertriglyceridemia in male MSG obese rats. In addition, we demonstrate that MSG hypothalamic obesity in fathers may program metabolic and vascular dysfunction in their progeny. As such, higher body fat accumulation in female offspring and reduced endothelium-dependent vasodilatation were observed in MSG descendants. Tau treatment in male founders did not prevent fat deposition in female MTAU offspring, whereas male MTAU progeny showed similar Ach-induced vasodilatation in

the thoracic aorta, indicating, for the first time, that this amino acid, when administered to MSG-hypothalamic obese fathers, may prevent vascular dysfunction in their offspring.

As expected, neonatal treatment with MSG efficiently increased perigonadal and retroperitoneal fat depots and induced hyperinsulinemia and hypertriglyceridemia, but lowered BW and body length in the paternal group. All these hypothalamic obesity characteristics have been reported previously (Balbo et al. 2000; Nardelli et al. 2011; Ribeiro et al. 2013, 2014). Although the mechanism of action that leads to obesity in MSG rodents is not completely understood, some reports suggest that neuroendocrine dysfunction and impaired fat metabolism may contribute to the manifestation of obesity (Hirata et al. 1997; Dolnikoff et al. 2001; Kim et al. 2005; Scomparin et al. 2009).

Several potential therapeutic and preventive actions of Tau against obesity development and its comorbidities have been reported (Nakaya et al. 2000; Nandhini et al. 2005; Tsuboyama-Kasaoka et al. 2006; Xiao et al. 2008; Ribeiro et al. 2012; Batista et al. 2013). Here, we confirm data regarding the preventive effect of Tau supplementation against body fat accumulation and hypertriglyceridemia in MSG rats (Nardelli et al. 2011). This effect may be related to the actions of Tau on lipid metabolism, as Tau supplementation is known to decrease circulating and hepatic levels of CHOL and TG in high-CHOL fed rats (Choi et al. 2006; Fukuda et al. 2011), and prevents adiposity in high-fat diet mice (Tsuboyama-Kasaoka et al. 2006; Batista et al. 2013) and in *Otsuka Long-Evans Tokushima Fatty* rats (Nakaya et al. 2000). Accordingly, overweight and obese non-diabetic subjects supplemented with 3 g Tau per day for 7 weeks exhibited decreased TG plasma levels (Zhang et al. 2004). These lower circulating TG levels were probably due to a suppression in hepatic TG secretion (Chen et al. 2004) and higher hepatic fatty acid oxidation, since Tau increases carnitine palmitoyltransferase 1 activity in the liver of high CHOL fed rats (Fukuda et al. 2011). The “antiobesity” effects of Tau may also occur in adipose tissue, since Tau enhances gene expression of several factors involved in energy expenditure in white adipose tissue (Tsuboyama-Kasaoka et al. 2006). This amino acid increases protein-kinase A activity in this tissue, enhancing lipolysis and decreasing the inhibitory action of insulin upon this process (Pina-Zentella et al. 2012).

The maternal metabolic condition as a determinant of the metabolic status in offspring has been extensively investigated (Cherif et al. 1996; Kalbe et al. 2005; de Campos et al. 2007; Catalano et al. 2009; Chavey et al. 2014). However, few studies have looked at the father’s role in such a process (Ng et al. 2010; Soubry et al. 2013; Fullston et al. 2013). Here, we verified that female MSG offspring showed larger fat depots (Table 3), despite demonstrating no modifications in BW, food consumption and glucose tolerance (Figs. 1 and 2 and Table 3). These findings are similar to those of a previous study in which female offspring from MSG mothers manifest obesity at 7 months of age (Campos et al. 2008), indicating that both male and female MSG parents may program fat accumulation in their progeny. Another report showed that offspring from fathers that were fed a high-fat diet did not show alterations in adiposity or plasma lipid levels, but rather demonstrated disruption of glucose homeostasis due to lower insulin secretion (Ng et al. 2010). As such, we suggest

that glucose intolerance and fat metabolism disruption can be observed in both male and female offspring from MSG fathers.

As discussed above, Tau has several actions that may prevent body fat accumulation; however, we observed that female MTAU offspring exhibit higher perigonadal and retroperitoneal fat stores (Table 3). These data suggest that although this amino acid improves lipid metabolism in MSG fathers, the effect is not propagated to the female progeny. Additionally, we demonstrated that Tau can impair fat metabolism in offspring, since female CTAU descendants also have larger fat stores (Table 3). Similar deleterious actions of Tau upon adiposity in progeny have been reported. Greater BW gain, body fat deposition and insulin resistance were evidenced in female offspring from dams supplemented with Tau (Hultman et al. 2007). Li et al. (2013) reported that, while Tau supplementation in pregnant rats submitted to an obesogenic diet normalized some hepatic inflammatory markers in female offspring, greater mortality was observed in neonates from control dams supplemented with Tau. These observations together with our results suggest that high Tau levels may have adverse effects on the preconceptional period and in normal pregnancy, changing fat metabolism in the offspring. It is possible that Tau modulates gene expression by epigenetic mechanisms.

In humans, maternal obesity and/or diabetes may predispose to cardiovascular dysfunction in offspring (Dabelea 2007; Reynolds et al. 2013; Marco et al. 2012; Gaillard et al. 2014). This metabolic imprinting in offspring has also been replicated in experimental models (Torrens et al. 2012; Turdi et al. 2013; Fan et al. 2013). Here, isolated thoracic aorta from male offspring of MSG-hypothalamic obese rats showed a clear impaired relaxation in response to Ach (Fig. 3). Remarkably, a decreased cholinergic action in mesenteric vascular beds from MSG-treated rats has been described (Lobato et al. 2011). Therefore, our results demonstrate that obesity and vascular dysfunction in MSG fathers involves an intergenerational mechanism. Previously, only the influence of maternal obesity on vascular dysfunction was reported. Male mice offspring from obese dams and those fed on a high-fat diet after weaning, showed lower Ach-induced relaxation in femoral arteries due to lower NO but higher superoxide production (Torrens et al. 2012). Similarly, isolated abdominal aorta from non-human primate (*Macaca fuscata*) offspring of obese mothers that consumed an obesogenic diet after weaning exhibited a decrease in Ach-induced relaxation, enhanced intima thickness and pro-inflammatory markers in the abdominal aortic wall (Fan et al. 2013).

We also evaluated in MSG offspring the effect of Tau supplementation given to the fathers on vascular reactivity. We verified that relaxation in aortas from MTAU offspring was similar to that observed in the CTL group (Fig. 3). Tau supplementation has several known benefits for cardiovascular function (Xu et al. 2008; Abebe and Mozaffari 2011). Tau supplementation decreases arterial blood pressure in experimental models of hypertension (Abebe and Mozaffari 2011). The amino acid also decreases basal contractile tone and induces relaxation in preconstricted arteries in an endothelium-dependent pathway (Ristori and Verdetti 1991). This Tau-mediated effect may occur due to activation of K^+ channels, since Tau-induced relaxation in renal and mesenteric arteries was abolished in the presence of tetraethylammonium, a non-selective K^+ channel blocker (Niu et al. 2008).

Tau may also contribute to vascular function in utero. Tau supplementation during gestation reduces blood arterial pressure in offspring of stroke-prone spontaneously hypertensive rats (Horie et al. 1987); this prenatal effect was associated with the antioxidant action of the amino acid (Lerdweeraphon et al. 2013). However, the ability of Tau supplementation during the preconception period to prevent vascular dysfunction in offspring was not observed. Thus, obesity is associated with changes in the gamete milieu, leading to alterations in spermatozoa viability, gene expression and DNA methylation (Ghanayem et al. 2010; Fullston et al. 2013). In addition, an altered imprint outcome was observed in neonatal children from obese fathers, indicating that epigenetic alterations during the preconceptional period, induced by life-style, malnutrition or obesity, may reprogram imprint marks during gametogenesis and early development (Soubry et al. 2013). Consequently, as Tau regulates the oxidative stress, motility and survival of the spermatozoa (Yang et al. 2010), we suggest that the high Tau milieu in MSG fathers may prevent epigenetic alterations in their gametes, possibly protecting against vascular dysfunction in the next generation of rats. However, an adverse effect on adiposity in female offspring may occur.

5 Conclusion

This study is the first report to demonstrate that MSG-hypothalamic obese fathers may determine body fat accumulation and vascular dysfunction in the next generation. Tau supplementation given to MSG-treated rats prevented the development of obesity, but this benefit was not propagated to female offspring. Normal aortic relaxation, in response to Ach, was present in MTAU offspring, indicating that this amino acid can prevent the intergeneration inheritance of cardiovascular dysfunction. Tau may act by regulating different epigenetic pathways in gametes, an effect that warrants further investigation.

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Taurine Supplementation Reduces Eccentric Exercise-Induced Delayed Onset Muscle Soreness in Young Men

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Abbreviations

ANOVA	Analysis of variance
AUC	Area under the curve
BEx	Before exercise
CIR	Upper arm circumference
CK	Creatine kinase
DOMS	Delayed onset muscle soreness
ECC	Eccentric exercise
Mb	Myoglobin
MIF	Maximal isometric force
ROM	Range of motion
ROS	Reactive oxygen spices
VAS	Visual analogue scale

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1 Introduction

Resistance exercise, particularly eccentric exercise (ECC), is a known method for maintaining muscle volume and strength (Miyachi 2013), and it has been recently recommended by major health organizations for health promotion and disease prevention (Pollock et al. 2000). It has been reported that only 30 min of ECC per week can improve quality of life (Paschalis et al. 2011).

It is generally known that unaccustomed exercise results in damage to the contracting muscles. Forced eccentric muscle contraction induces severe muscle soreness and damage (Clarkson et al. 1992). Symptoms of muscle soreness usually present hours or even days after exercise and are termed as delayed onset muscle soreness (DOMS) (Armstrong et al. 1991). Severe muscular contractions increase reactive oxygen species (ROS) production, which has been reported to be partially associated with DOMS and muscle damage (Close et al. 2004).

Taurine (2-aminoethylsulfonic acid), a sulfur-containing amino acid, is found in high concentration within several mammalian tissues and organs, especially in skeletal muscle. Previous studies have shown that taurine has a protective effect against oxidative stress; taurine supplementation is shown to reduce exercise-induced oxidative stress (Zhang et al. 2004; Close et al. 2004).

Accordingly, the purpose of the present study was to determine whether taurine supplementation reduces DOMS and muscle damage after high-intensity ECC.

2 Methods

2.1 Participants

Twenty-nine recreationally active young men (age, 25.3 ± 0.1 years; body mass, 66.2 ± 0.9 kg; height, 174.6 ± 0.1 cm) were recruited for the present randomized, double-blind study. None of the participants had undergone any regular resistance training prior to the present study. Participants were instructed to fast overnight, avoid vigorous physical activity, and abstain from drinking caffeine and alcohol for at least 12 h before each measurement. The present study was approved by the Human Subjects Committee of the University of Tsukuba. All participants provided written informed consent prior to initiation of the present study.

2.2 Experimental Protocol

Participants were randomly divided into the two groups: placebo supplement group (n = 14) and taurine supplement group (n = 15). Before starting supplementation, body mass, height, and maximum isometric force of elbow flexor were measured by

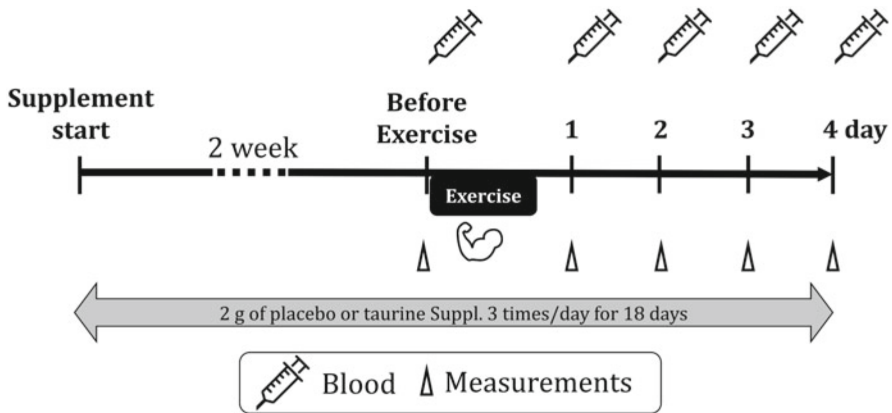

Fig. 1 Schematic illustrating the experimental protocol. Abbreviations: Blood, blood sampling; Measurements: determination of muscle soreness, upper arm circumferences, and elbow range of motion

a dynamometer (Biodex system 3, SAKAI Medical. Co., Ltd.). After 2 weeks of supplementation, all participants performed ECC of the elbow flexor. We measured each parameter (described later) before exercise (BEx) and for 4 days after exercise.

2.3 Supplement Protocol

Participants orally consumed 2 g of placebo (lactose) or taurine (Taisho Pharmaceutical Co., Ltd., Tokyo, Japan) powder three times a day after meals for 2 weeks before exercise (Fig. 1). All participants were instructed to fill out a supplemental checklist after each supplement. Supplementation was continued in a double-blind manner until the third day after exercise. At the same time, participants were instructed to continue their normal food intake, that is not to change the amount or frequency of dietary meat, fish or shellfish intake, and not to use any dietary supplements, anti-inflammatory medications, or drugs that could affect muscle soreness and damage until the end of the study.

2.4 Exercise Protocol

Participants performed two sets of maximal eccentric unilateral contractions of the elbow flexor muscle, each set consisting of 20 contractions while seated upright on a dynamometer, as previously described with minor modifications (Barnes et al. 2010). The exercise was performed with the non-dominant arm. Eccentric

contractions consisted of maximal contraction through a range of motion (ROM) from 90° to 180° of elbow flexion. Each contraction was held for 3 s and repeated every 9 s with a 4 min period of rest between sets.

2.5 Measurements

2.5.1 Measurement of Delayed Onset Muscle Soreness

Subjective assessment of elbow flexor soreness in the biceps brachii muscle was surveyed using the visual analogue scale (VAS), which consisted of a 100-mm line with “no pain” at one end and “extreme pain” at the other end (Ra et al. 2013), to assess pain perceived during palpation of the elbow flexors. VAS scores were measured before exercise and for 4 days after exercise (Fig. 1).

2.5.2 Measurement of Upper Arm Circumference

The upper arm circumference (CIR) was used as an indirect indicator of muscle damage. It was measured BEx and for 4 days after exercise (Fig. 1). The CIR was measured at five points located 3, 5, 7, 9, and 11 cm proximal to the elbow joint on a relaxed arm in the standing position using constant-tension tape. To avoid daily variations in the measurement position, sites on the upper arm were marked using a semi-permanent ink pen during the first testing session. Each CIR was measured in duplicate and the mean value was used for analysis. The values obtained during the 4 days after exercise were presented as differences from the baseline values.

2.5.3 Measurement of Elbow Range of Motion

The elbow ROM was measured using a goniometer while the participants stood with the elbow held in full flexion and full extension. These elbow angles were measured in duplicate and the mean value was used for analysis. The values measured on the 4 days after exercise were presented as the differences from the values before exercise.

2.5.4 Serum Markers of Muscle Damage

Blood samples were collected from the antecubital vein at five different time points, before exercise and for 4 days after exercise (days 1–4) (Fig. 1). On the exercise day, blood was collected before supplement intake and exercise. During 4 days after exercise, blood was collected at 7:00 a.m. before breakfast and supplement intake. Serum was centrifuged for 30 min after the formation of a solid clot. Serum

myoglobin (Mb) and creatine kinase (CK) levels were analyzed and used as indicators of muscle damage, as described in the Japan Society of Clinical Chemistry consensus methods.

2.6 Statistic Analysis

Data are presented as means ± SE. Changes in measurements were tested for effects of time and group in a two-way repeated measures analysis of variance (ANOVA). If a significant F value was obtained, then post hoc analysis was performed using the Bonferroni method with statistical significance set at $p < 0.05$. The area under the curve (AUC) was calculated as the sum of four trapezoid areas separated by each measurement time point. Analysis was conducted using SPSS software version 21.0 for Windows (SPSS Japan Inc.).

3 Results

3.1 Delayed Onset Muscle Soreness

The ECC protocol was effective in causing DOMS in participants, as indicated by significant increases in VAS scores (Fig. 2). DOMS was significantly attenuated by taurine supplementation (Fig. 2a). The AUC of DOMS was lower in the taurine supplemented group than in the placebo group (Fig. 2b).

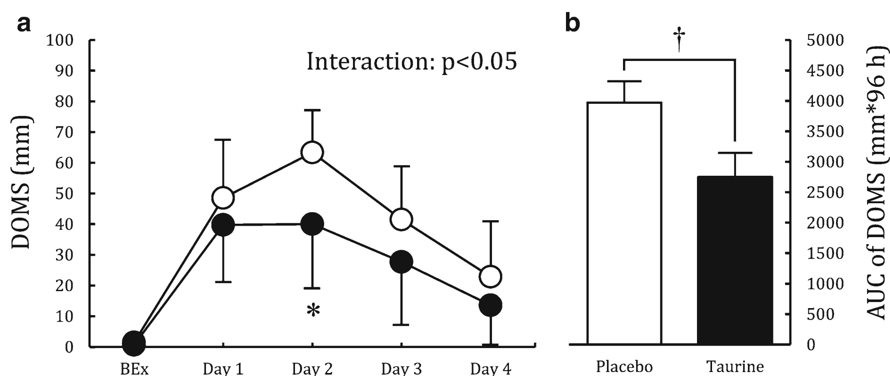

Fig. 2 DOMS score is plotted along the time sequence (**a**) and the sum of the DOMS score during experiments is presented (**b**) for both placebo and taurine supplementation groups (*open* and *closed circle*, respectively). *$p < 0.05$ compared to the placebo group by Bonferroni post hoc analysis after two-way repeated measures ANOVA. †$p < 0.05$ compared to the placebo group by unpaired student's *t* test. *AUC* area under the curve, *DOMS* delayed onset muscle soreness, *VAS* visual analogue scale

3.2 Parameters of Muscle Damage

3.2.1 Upper Arm Circumference

The CIR increased gradually and significantly until the end of the experiments in both groups. As shown Fig. 3a, changes in CIR were smaller in the taurine supplemented group, but not significantly different between the two groups throughout the study.

3.2.2 Elbow Range of Motion

In both groups, peak reduction in ROM was observed from day 1 to day 2 after ECC, and gradually recovered at the end of the study. However, no significant differences were observed between the two groups (Fig. 3b).

3.2.3 Serum Myoglobin Concentration and Creatine Kinase Activity

Serum Mb concentration and CK activity were within normal reference ranges before exercise without any significant difference between the two groups. After exercise, serum Mb concentration and CK activity tended to be elevated in the taurine group but there was no significant difference between the groups (Fig. 3c, d).

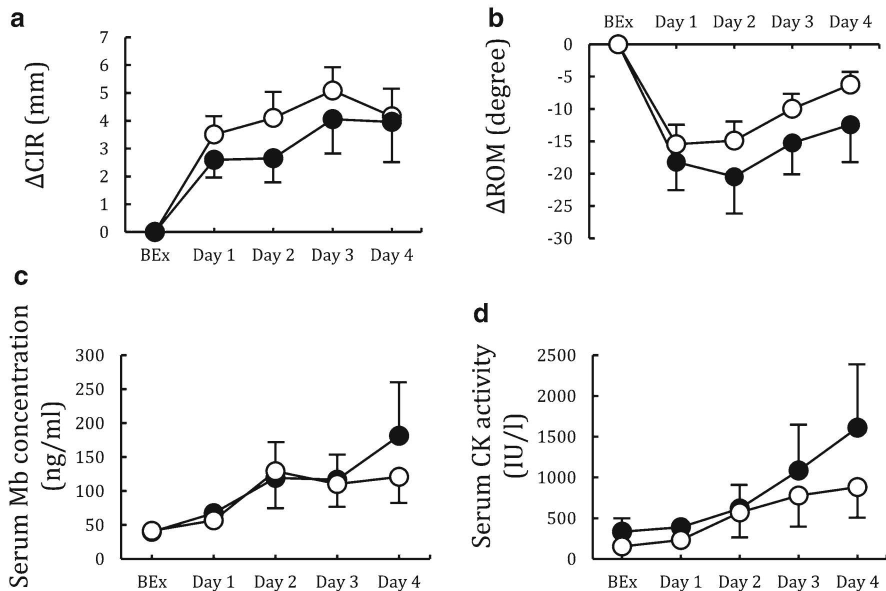

Fig. 3 Changes in CIR (**a**), ROM (**b**), serum Mb concentration (**c**) and CK activity (**d**) plotted along the time sequence for both the placebo and taurine supplementation groups (*open* and *closed circle*, respectively). *CIR* upper arm circumference, *ROM* elbow range of motion, *Mb* myoglobin, *CK* creatine kinase

4 Discussion

We investigated whether taurine supplementation prevents DOMS and muscle damage after high-intensity ECC. Our results indicated that continuous taurine supplementation significantly reduces the severity of DOMS induced by elbow ECC in healthy young men.

DOMS occurs after unaccustomed exercise and is associated with ECC. Several studies have already reported that ECC increases oxidative stress (Close et al. 2004; Silva et al. 2011; Lee et al. 2002). In the present study, taurine supplementation was shown to reduce the severity of DOMS after ECC. It is possible that increasing ROS production was attenuated by taurine supplementation. In fact, taurine supplementation has been reported to decrease the production of oxidative stress. da Silva et al. (2014) demonstrated that taurine supplementation prevents increases in carbonyl protein and lipoperoxide levels.

Eccentric types of muscle contraction, where muscles are lengthened during force generation, have been shown to cause muscle damage. In the present study, high-intensity elbow ECC tended to cause changes in the parameters indicative of muscle damage (CIR, ROM, serum CK activity and Mb level). Although taurine supplementation tended to attenuate changes in these parameters, there were no significant differences among these parameters between the two groups. Several studies have reported that a reduction in maximal isometric force (MIF) is the most common initial injury phenomenon following ECC (Faulkner et al. 1993). However, we did not measure the changes in MIF before and after high-intensity ECC. Therefore, further studies are required to assess whether taurine supplementation can attenuate the reduction of muscle force after ECC.

Our present experimental protocol has some limitations. First, data on changes in oxidative stress during the experiments (e.g., ROS activity and anti-oxidative stress enzymes levels) were not obtained. Second, we continuously administered taurine supplementation to determine the effects of taurine supplementation on DOMS and muscle damage after ECC. Therefore, we could not ascertain the optimal timing of taurine supplementation to reduce DOMS and muscle damage after ECC. Thus, it is important to determine the timing of taurine supplementation most effective in reducing DOMS after ECC in future studies. Third, we didn’t measure 24-h urinary taurine levels as an index of dietary taurine intake. Thus, we couldn’t clarify the effect of dietary taurine intake on the results of our present study. In order to clarify the effects of dietary taurine intake on the DOMS and muscle damage after ECC, we need to measure urinary taurine concentration in future studies.

5 Conclusion

In conclusion, our results suggest that continuous taurine supplementation can reduce DOMS after high-intensity ECC in healthy young men.

Acknowledgements We would like to thank Dr. Ryuichi Ajisaka, Dr. Yoshio Nakata, and Dr. Nobutake Shomojo for scientific and technical support. We also thank Mr. Ryota Higashino for providing the blood sample collected from the participants. The present study was supported by a grant from Taisho Pharmaceutical Co. Ltd., (Tokyo, Japan) and a Grant-in-Aid for the Japan Society for the Promotion of Science (JSPS) Fellows.

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The Effects of Taurine Administration Against Inflammation in Heavily Exercised Skeletal Muscle of Rats

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Abbreviations

CD68	Cluster of differentiation 68
EDL	Extensor digitorum longus
IHC	Immunohistochemistry
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
SOL	Soleus
Tau	Taurine
TNF-α	Tumor necrosis factor-α

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1 Introduction

Taurine (2-aminoethanesulfonic acid) is the most abundant amino acid in the body. It is found in high concentrations in most tissues, including skeletal muscle, heart, nerve, brain, and liver in vertebrates (Jacobsen and Smith 1968). Its cytoprotective activity against tissue damage involves several essential biological, physiological, and pharmacological mechanisms, including membrane stabilization (Pasantes-Morales et al. 1985), antioxidation (Gordon and Heller 1992; Sugiura et al. 2013) and osmoregulation (Thurston et al. 1980). Because of the absence of taurine biosynthesis, the large intracellular taurine pool of skeletal muscle is maintained by a specific transporter (Ramamoorthy et al. 1994). The uptake of taurine differs with muscle type (Kim et al. 1986), as the steady state level of taurine concentration of the slow-twitch fiber is higher than that of the fast-twitch fiber (Iwata et al. 1986). Heavy exercise increases oxidative stress and damages skeletal muscle (Sugiura et al. 2013). The damage is characterized by enhanced muscle protein catabolism and acute inflammation, followed by muscle tissue regeneration (Meeson et al. 2004; Ciciliot and Schiaffino 2010). The progression of muscle repair is modulated by essential and semi-essential amino acids and involves the modulation of inflammation and protein anabolism (Dort et al. 2013; Miyazaki et al. 2004). Taurine protects skeletal muscle against intense exercise-induced nitrosative inflammation and ensuing DNA damage by preventing iNOS expression and the increase in nitrosative stress mediated by heavy exercise (Sugiura et al. 2013). Therefore, the regulation of inflammation is likely to differ with muscle type. To evaluate differences related to muscle type, we observed the effect of taurine administration on inflammation in heavily exercised skeletal muscle of rats.

2 Methods

2.1 Ethics Statement

All experimental protocols were approved by the Animal Ethics Committee of Suzuka University of Medical Science, Japan.

2.2 Animal and Experimental Design

Eight-weeks-old male Wistar rats were obtained from SLC (Hamamatsu, Japan) and weighed 250–260 g on arrival. They were housed in cages (Max, six per cage) and maintained on water and food *ad libitum*. All animals were maintained at 24.0 °C (45–55 % of humidity) with a 12 h light and 12 h dark cycle. The 18 rats were randomly divided into three groups; Exercise plus saline group (n = 6), Exercise plus taurine group (n = 6), and Control group (n = 6).

2.3 Exercise Protocol and Taurine Supplementation

The exercise protocol of the present study used a widely recognized animal model to study post exercise inflammatory responses. We designed a heavy treadmill exercise protocol on a motor-driven tread-mill (model MK-680; Muromachi Kikai, Tokyo, Japan) in which rats began exercising at 8 m/min for 10 min during the week preceding the actual exercise experiments. The rats became accustomed to locomotion in preparation of the final exercise experiments without stimulating development of skeletal muscle as an adaptation to training. Each group ran on the treadmill at 20 m/min, 25 % grade, for 20 min or until exhaustion. Exhaustion was determined as the point when an animal was unable to right itself when placed on its side. The workload was ~75 % of the rat's maximal aerobic capacity. Rats subjected to the exercise protocol were either treated with saline or taurine (intra-abdominally) 1 h prior to the onset of the exercise protocol.

2.4 Surgical Method and Muscle Collection

In the control group, extensor digitorum longus and soleus muscles of both legs were removed in the morning of the 10th day. Then, muscle tissue was formalin-fixed and the samples paraffin-embedded.

In the exercise plus saline group and the exercise plus taurine group, the animals were euthanized by cardiac exsanguination under anesthesia 48-h after the 10th day of exercise, and the muscles were immediately excised. The protocol was designed to restore basal conditions prior to sacrifice.

2.5 Immunofluorescence Study of TNF-α, CD68, and IL-6

Standard immunofluorescence methods were used to examine the distribution of TNF-α, CD68, and IL-6 in muscle tissue and normal controls. After deparaffinization and rehydration, antigen was retrieved in 5 % urea by microwave heating for 5 min and then incubated in 1 % H_2O_2 for 30 min to block endogenous peroxidase activity. Sections of 5 μm thickness were incubated overnight at room temperature with the following antibodies: TNF-α goat polyclonal antibody, CD68 mouse monoclonal antibody, and IL-6 rabbit polyclonal antibody (1:200, Santa Cruz Biotechnology Co., Ltd). For the goat antibodies (TNF-α), the sections were incubated with Donkey anti-goat IgG-Alexa 594 for 2 h. For mouse antibody (CD68), the sections were incubated with Donkey anti-mouse IgG-Alexa 488 for 2 h. For the rabbit antibodies (IL-6), the sections were incubated with Goat anti-Rabbit IgG-Alexa 594 for 2 h.

2.6 Immunohistochemical Grading

Immunohistochemical (IHC) grading, which is based on intensity and frequency of staining, was performed by two independent investigators. The staining intensity was scored as negative (−), weak (+), moderate (++), strong (+++), or very strong (++++). In addition, we measured the brightness of the image by using ImageJ (Wayne Rasband) (Schneider et al. 2012).

2.7 Statistical Analysis

Data are presented as means ± SEM. The two-tailed Student's *t*-test was performed. Differences were considered statistically significant at $P < 0.05$.

3 Results

3.1 Body Weight Progression and Consumption of Water and Food

Over the duration of the experiments, there was no significant difference in body weight progression between the Control and Taurine groups. All rats were in good health, with no pathological signs observed throughout the period of study.

3.2 Localization of TNF-α, IL-6 and CD68 in Normal and Exercised EDL and SOL Skeletal Muscle

To observe TNF-α, IL-6 and CD68 expression, immunohistochemistry was performed on normal and exercised skeletal muscle. TNF-α and IL-6 immunofluorescence staining shows intense immunoreactivity in the stromal cell of endomysium and blood vessels of EDL and SOL muscle (Figs. 1, 2, 3, and 4). In contrast, CD68 immunoreactivity was localized in some of the muscle fibers of EDL and SOL (Figs. 1, 2, 3, and 4).

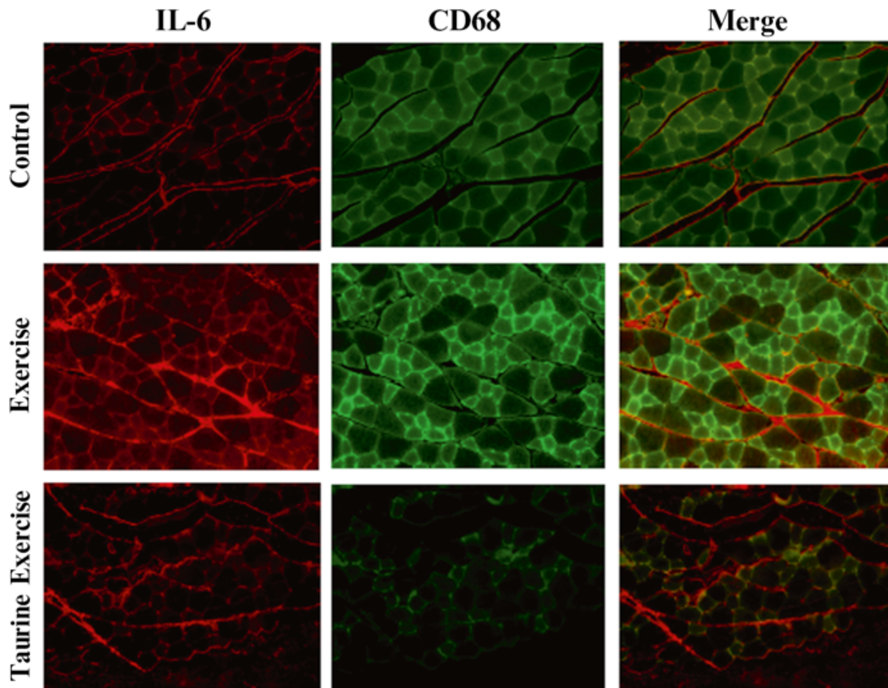

Fig. 1 Expression of IL-6 and CD68 in EDL of each experimental group (Control, Exercise plus saline, Exercise plus taurine). The expression of IL-6 and CD68 in EDL of the Exercise plus taurine administration group shows low immunoreactivity compared to that of the Exercise plus saline group

3.3 Comparative Quantification of TNF-α, IL-6 and CD68 Immunoreactivities in EDL and SOL Skeletal Muscle

To compare the expression of TNF-α, IL-6 and CD68 in different skeletal muscle of each group, immunoreactive signal intensity was quantified by grading. TNF-α and IL-6 signal intensities exhibited a definitive trend in EDL that was less apparent in SOL. There was no difference in CD68 expression between EDL and SOL (Tables 1 and 2). In the heavy exercise group, TNF-α, IL-6 and CD68 signal intensities significantly increased compared to that of the control group and exercise plus taurine group ($p < 0.05$) (Figs. 1, 2, 5 and 6).

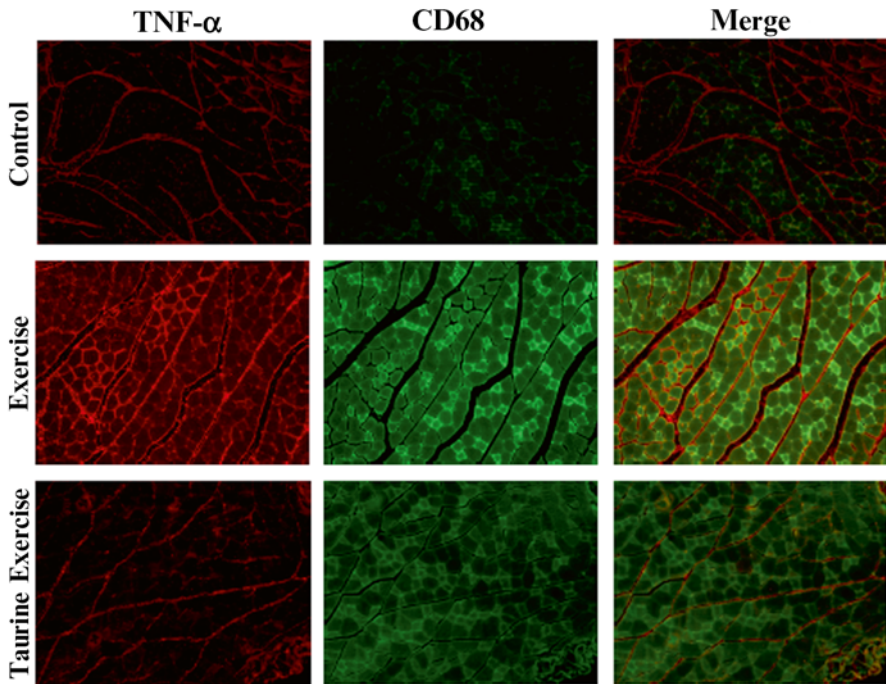

Fig. 2 The expression of TNF-α and CD68 in EDL of each group. The expression of TNF-α and CD68 in EDL of the Exercise plus taurine administration group shows low immunoreactivity compared to that of the Exercise plus saline group

4 Discussion

In the present study, we compared the effects of taurine administration on inflammation of various muscle types in rats subjected to a heavy exercise protocol. Our study revealed three findings. First, most of TNF-α and IL-6 preferentially accumulate in stromal cells of skeletal muscle. In contrast, CD68 accumulates within the muscle fibers. Second, signal intensity for TNF-α and IL-6 was observed in some muscle fibers after heavy exercise. Finally, taurine treatment reduced measures of inflammation in rat skeletal muscle after exercise injury. Skeletal muscle damage leads to changes in tissue morphology and function that may last for several weeks (Lapointe et al. 2002; Smith et al. 2008). Heavy exercise is characterized by muscle protein catabolism and acute inflammation, followed by muscle regeneration (Meeson et al. 2004). The progression of muscle repair is modulated by essential and semi-essential amino acids, which alter inflammation and protein anabolism (Miyazaki et al. 2004; Dort et al. 2013). After muscle injury, myocytes and other cells release a number of cytokines, including IL-1β, IL-6, IL-8 and TNF-α, which cause neutrophils to produce a host of cytotoxic substances, including reactive

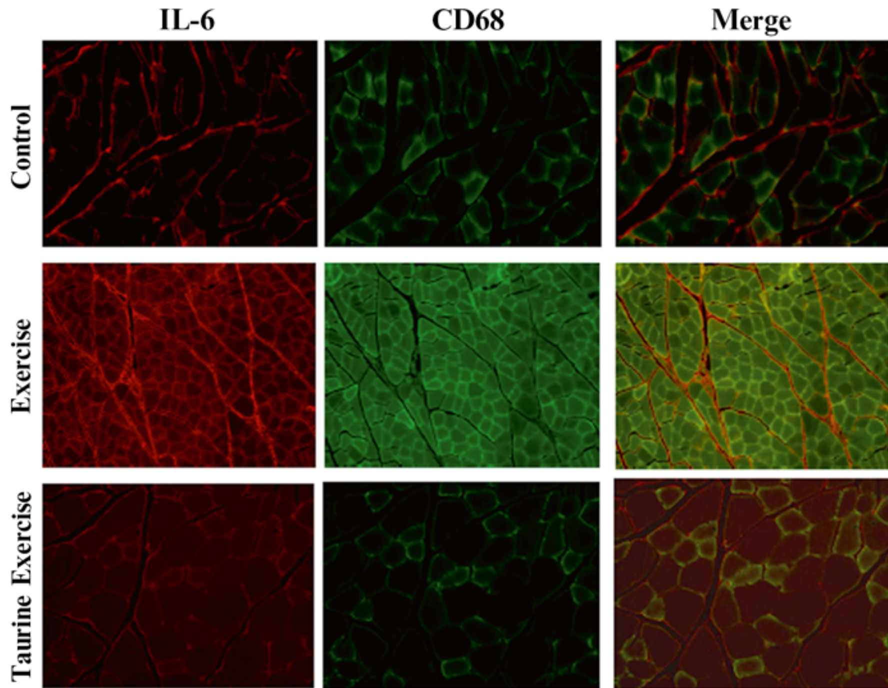

Fig. 3 The expression of IL-6 and CD68 in SOL of each group. The expression of IL-6 and CD68 in SOL of the Exercise plus taurine administration group shows low immunoreactivity compared to that of the Exercise plus saline group

oxygen species, such as superoxide anions, hypochlorite, and hydrogen peroxide (Best et al. 1999; Brickson et al. 2001). The cytokines IL-1, IL-6, and TNF-α stimulate pathways that contribute to activation of the enzyme NADPH-oxidase, which in turn generates reactive oxygen species. Previous research reported that taurine may exert various biological actions that contribute to muscle repair, such as improved outcome of an inflammatory insult, owing to its ability to decrease the production of major pro-inflammatory cytokines (TNF-α, IL-6) (Pilon et al. 2011; Rudkowska et al. 1995). Although the mechanism by which taurine reduces inflammation in skeletal muscle is not fully understood, taurine plays an important role in cytoprotection against ischemia and hypoxia in smooth muscle of stomach (Ma et al. 2003). Taurine efflux may also contribute to the regulatory volume decrease mediated by ion channels and triggered as a response to cell swelling. Released taurine can in turn react with $HOCl^-$ produced by activated leukocytes to form taurine chloramine, a strong reactant that inhibits the production of TNF-α and other proinflammatory mediators (Barua et al. 2001).

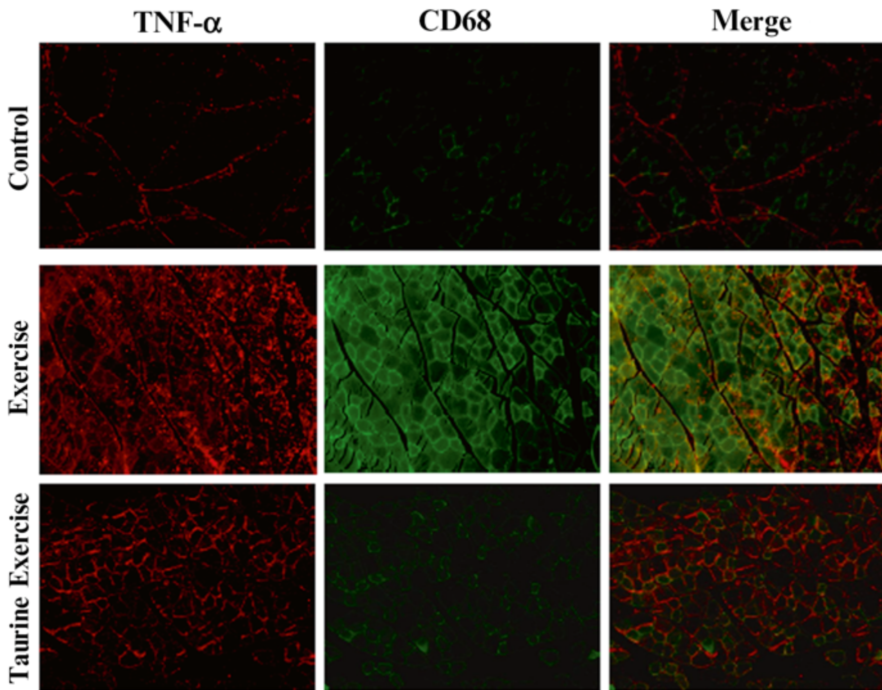

Fig. 4 The expression of TNF-α and CD68 in SOL of each group. The expression of TNF-α and CD68 in the Exercise plus taurine administration group show low immunoreactivity compared to that of the Exercise plus saline group

Table 1 The effect of exercise and taurine treatment on the immunoreactivities of IL-6, TNF-α and CD68 of EDL. Immunohistochemical (IHC) grading based on intensity and frequency of staining was performed by two independent investigators

EDL				
Biomarkers	Location	Control	Exercise plus saline	Exercise plus taurine
TNF-α	Stromal cell	+	+++	+
	Muscle fibers	–	–	–
IL-6	Stromal cell	+	++++	+
	Muscle fibers	–	–	–
CD68	Stromal cell	–	–	–
	Muscle fibers	+	+++	+

In a previous study by our laboratory, we found that taurine administration significantly reduces iNOS expression, indicating that heavy exercise-induced nitrosative and oxidative stress is modulated by taurine. Up-regulation of iNOS in skeletal muscle is responsible for nitrosative muscle damage induced by heavy exercise, a consequence of inflammation-mediated activation of NF-κB signaling (Sugiura

Table 2 The effect of exercise and taurine treatment on the immunoreactivities of IL-6, TNF-α and CD68 of SOL. Immunohistochemical (IHC) grading based on intensity and frequency of staining was performed by two independent investigators

SOL				
Biomarkers	Location	Control	Exercise plus saline	Exercise plus taurine
TNF-α	Stromal cell	+	++	+
	Muscle fibers	–	–	–
IL-6	Stromal cell	+	+++	+
	Muscle fibers	–	–	–
CD68	Stromal cell	–	–	–
	Muscle fibers	+	+++	+

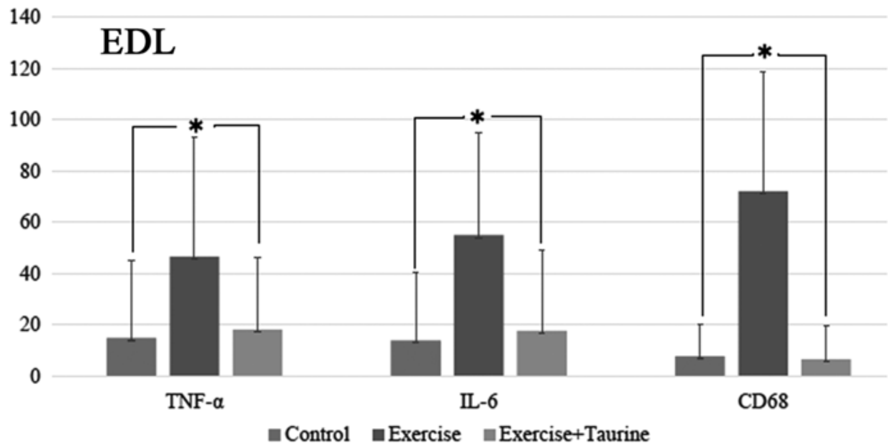

Fig. 5 The average immunoreactivities of IL-6, TNF-α and CD68 in EDL. TNF-α, IL-6 and CD68 immunoreactivities significantly increased in rats undergoing heavy exercise. The immunoreactivities significantly decreased in the taurine exercise group compared to the heavy exercise group. Result show Mean ± SEM, for n = 6 rats in each experimental group. $^*P < 0.05$, exercise group versus exercise + taurine group or control group. The two-tailed Student's *t*-test

et al. 2013). ATP production and oxygen supply to mitochondria needs to be increased to activate skeletal muscle during exercise. An increase in oxygen consumption likely means an increase in the generation of superoxide radicals (O_2^-). Taurine appears to reduce the production of O_2^- via redox reaction, particularly in places with high production of O_2^-, such as mitochondria (Hansen et al. 2006). This involves the conjugation of taurine with $tRNA^{Leu(UUR)}$, which increases the biosynthesis of ND6, a subunit of complex I of the electron transport chain, and prevents the diversion of electrons to oxygen forming O_2^- (Jong et al. 2012). Type I muscle fibers contain a large number of mitochondria and perform aerobic energy metabolism by consuming a large number of oxygen molecules to produce ATP during

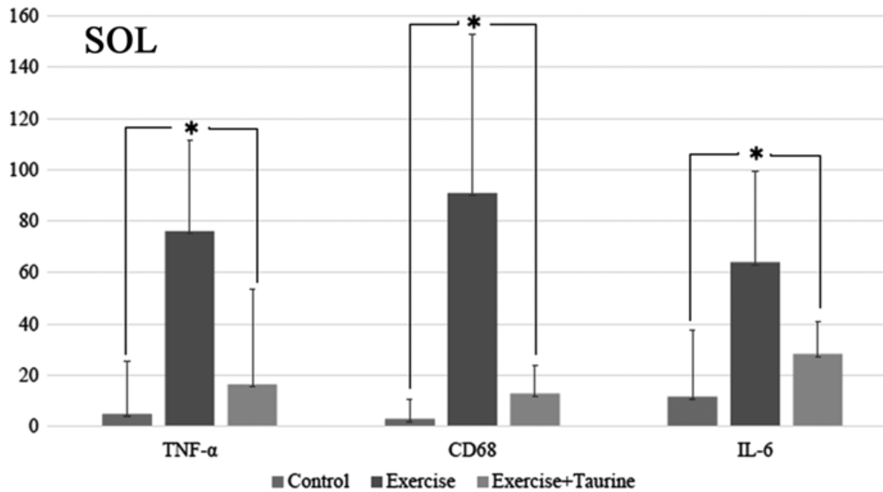

Fig. 6 The average immunoreactivity of IL-6, TNF-α and CD68 in SOL. TNF-α, IL-6 and CD68 immunoreactivities significantly increased in rats performing heavy exercise. Those immunoreactivities significantly decreased in the taurine exercise group compared to that of the heavy exercise group. Result show Mean ± SEM, for n = 6 rats in each experimental group. *P < 0.05, exercise group versus exercise + taurine group or control group. The two-tailed Student's *t*-test

exercise. Therefore, it is likely that type I muscle fibers generate a high level of free radicals from oxygen and are vulnerable to oxidative and nitrosative stress. Compared with type II muscle fibers, type I muscle fibers contain a higher level of polyunsaturated fatty acid and are reportedly vulnerable to lipid peroxidation (Nikolaidis and Mougios 2004; Nikolaidis et al. 2006) as well as to other types of oxidative damage. Because type I muscle fibers also contain a higher number of mitochondria (Moyes 2003) than type II muscle fibers, they may produce a higher level of free radical production during exercise and even at rest.

5 Conclusion

In conclusion, the present study demonstrates that taurine therapy protects against intense exercise-induced inflammation. Taurine may have various biological functions in muscle repair, such as an improved resolution of inflammation, owing to its ability to decrease the production of major pro-inflammatory cytokines.

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Needs Assessment for Development of Health Functional Taurine-Containing Food for Korean College Students

Su Bin Na, Chae Ryung Ha, So Hyun Kim, Jeong Soon You, So Young Kim, and Kyung Ja Chang

Abbreviations

CAN-pro	Computer aided nutritional analysis program
HFF	Health functional food
SE	Standard error
SPSS	Statistical Product and Service Solution

1 Introduction

HFFs are processed foods made using ingredients or raw materials serving useful functions in the human body (Yun et al. 2008). A variety of HFFs have been developed and sold in an effort to minimize degradation of physical function (Kang et al. 2011). HFFs have generated considerable interest by the health and food industries, as they serve a tertiary function for foods all over the world (Hel et al. 2006).

Dietary taurine (2-aminoethane sulfonic acid) intake may play important physical and psychological roles (Padantes-Morales and Cruz 1984; Gaull et al. 1985; Yokogoshi et al. 1999; Nakaya et al. 2000). In many studies, taurine has been reported to possess various biological activities, such as anti-obesity, antioxidant, hypoglycemic, and hypolipidemic effects (Gaull et al. 1985; Kim and Park 2002). Therefore, the need for development of taurine-containing HFF is increasing.

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Many studies on perceptions and consumption of HFF have been reported (Kim 2010; Kang and Lee 2010; Ohn and Kim 2012); however, studies regarding health functional taurine-containing food have been limited. Therefore, we investigated perception and needs assessment regarding HFF for the development of health functional taurine-containing food in Korean college students.

2 Methods

2.1 Subjects and General Characteristics

The subjects were college students in Incheon, Korea who attended a nutrition-related non-major class via Internet. A total of 251 subjects were included: 162 males (64.5 %) and 89 females (35.5 %).

General characteristics examined included age, type of residence, and availability of pocket money.

2.2 Needs Assessment Regarding HFF

Surveys were conducted using questionnaires. The questionnaires included perception and consumption status of HFF, and functions of HFF needed.

2.3 Dietary Taurine and Nutrient Intake Assessment

A 3 day-recall method was used for assessment of dietary intake (2 weekdays and 1 weekend day). Dietary taurine and nutrient intakes were estimated using a computer aided nutritional analysis program (CAN-pro 3.0, The Korean Nutrition Society, Korea), which contained a taurine content database for 17 food groups.

2.4 Statistical Analysis

Statistical analysis was performed using the Statistical Product and Service Solution (SPSS) 20.0 program. The frequency, percentages, means, and standard error (SE) were determined. The chi-square test and Student *t*-test were performed for determination of significant differences.

3 Results

3.1 General Characteristics

General characteristics of the subjects are shown in Table 1. The average age of males was 23.0 years and that of females was 21.1 years. The reason male subjects were older than the female subjects related to the needs of students returning to school after military service; 91 male subjects (56.2 %) and 65 female subjects (73.0 %) lived with families and prepared their own meals while a smaller number lived in dormitories; 102 male subjects (63.0 %) and 54 female subjects (60.7 %) spent 195–390 dollars per month as pocket money, with fewer spending less than 195 and even fewer subjects spending more than 390. There were significant differences in age ($p<0.001$) and residence ($p<0.05$) according to gender.

3.2 Consumption of HFF

Among the male subjects, 65 (40.1 %) consumed HFF while 97 (59.9 %) did not. Of the 89 female subjects, 43 subjects (48.3 %) consumed HFF and 46 subjects (51.7 %) did not. The type of HFF consumed by the subjects is shown in Fig. 1. Most HFF intake among males was vitamins and minerals (35.8 %), red ginseng (34.9 %), protein (10.1 %), and omega-3 fatty acid (8.2 %). Among females, most HFF intake was vitamins and minerals (47.4 %) and red ginseng (32.1 %), with consumption being less frequent for omega-3 fatty acid (7.7 %) and individually approved functional ingredients. In comparison with other studies, the frequency of HFF consumption was 33.6 %, which was lower than the rate of consumption of HFF by college students surveyed in Korea (Kim 2010).

Table 1 General characteristic of the subjects

Variables	Male (n = 162)	Female (n = 89)	t-value or χ^2-value
Age (years)	23.0 ± 0.2[a]	21.1 ± 0.2	7.079***[c]
Type of residence			
Living with family	91 (56.2)[b]	65 (73.0)	2.111*
Preparation of own meals	48 (29.6)	17 (19.1)	
Dormitory or etc.	23 (14.3)	7 (7.9)	
Pocket money (USD)			
≤195	34 (21.0)	19 (21.3)	−0.047
195–390	102 (63.0)	54 (60.7)	
≥390	26 (16.0)	16 (18.0)	

[a]Means ± SE
[b]n (%)
[c]Superscripts are significantly different between gender by Student t-test or chi-square test (*$p<0.05$, **$p<0.01$, ***$p<0.001$)

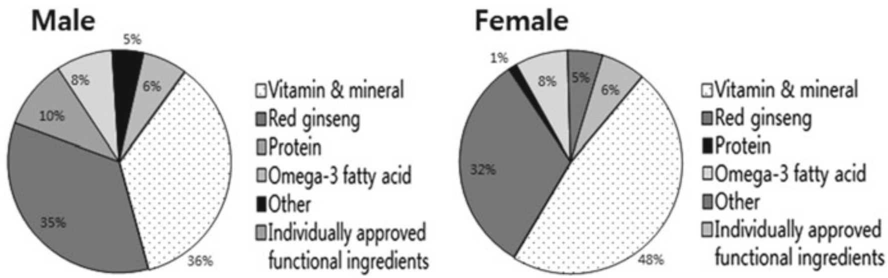

Fig. 1 Kinds of HFF consumed by the subjects. The most widespread HFF consumed by males was vitamins and minerals, followed by red ginseng, protein, and omega-3 fatty acids. Among females, the order of HFF intake was vitamins and minerals, red ginseng, omega-3 fatty acids, and individually approved functional ingredients

3.3 Needs Assessment Regarding HFF

Needs assessment regarding HFF is shown in Table 2. Need for HFF function is shown in Table 2. The functions of HFF used by the subjects included control of body fat (male 22.2 % and female 24.7 %), enhancement of immunity, and health of the gut, in that order. Taurine supplementation decreased fat storage in the abdominal cavity of broiler chicks (Kim and Park 2002). The reliability of the health effects of functional health foods was found to influence the expectations when subjects consume HFF. In the case of the consumer group, an expectation of health due to the various effects of HFF was higher than in the non-consumer group (Kim et al. 2001; Kim and Keen 2002).

Sixty three male subjects (38.9 %) and 48 female subjects (53.9 %) preferred specialty stores for HFF. Most subjects chose to buy HFF in a specialty store to ensure a reliable product (male 61.9 % and female 70.8 %) with a secondary reason being available and various product systematic distribution networks and the fewest subjects choosing the specialty store because of its proximity to the residence.

Ninety two male subjects (56.8 %) and 63 female subjects (70.8 %) preferred a specialized consultant for HFF. Most subjects wanted to buy HFF from a specialized consultant for HFF in order to receive accurate information on the product (male 54.3 % and female 50.8 %), with other reasons according to the order of preference being dissemination of exact knowledge, understanding of functional ingredients and learning scientific characteristics of the products and for reliability of the consumer.

3.4 Dietary Taurine and Nutrient Intake

The average dietary taurine and nutrient intakes are shown in Tables 3 and 4. Among males, the average dietary intake of taurine was significantly less in the consumption group (139.2 mg/day) than in the non-consumption group (171.0 mg/day).

Table 2 Needs assessment of HFF

Variables	Male N (%)	Female N (%)	χ^2-value
Need of HFF functions			
Control of body fat	36(22.2)	22(24.7)	4.594
Enhancement of immunity	33(20.4)	19(21.4)	
Health of bone, joint, cartilage, tooth	16(9.9)	12(13.5)	
Health of the gut	14(8.6)	9(10.1)	
Maintain blood pressure and blood sugar	10(6.2)	6(6.7)	
Blood cholesterol regulation	11(6.8)	4(4.5)	
Get rid of free oxygen radicals	7(4.3)	5(5.6)	
Others	35(21.6)	12(13.5)	
Need of specialty store			
Yes	63(38.9)	48(53.9)	8.603*[a]
No	99(61.1)	41(46.1)	
Reason for need of specialty store			
Reliability of products	39(61.9)	34(70.8)	1.406
Various product purchase	13(20.6)	6(12.5)	
Systematic distribution network	8(12.7)	6(12.5)	
Purchase nearby	3(4.8)	2(4.2)	
Need of specialty consultant			
Yes	92(56.8)	63(70.8)	8.063*
No	709(43.2)	26(29.2)	
Reason for need of specialty consultant			
Information offering of the products	50(54.3)	32(50.8)	3.344
Disseminate exact knowledge	28(30.4)	22(34.9)	
Understanding of functional ingredient	6(6.5)	7(11.1)	
Scientific characteristic of products and reliability of consumers	6(6.5)	1(1.6)	

[a]Superscripts are significantly different between gender by chi-square test (*$p<0.05$)

Among females, the average dietary intake of the taurine consumption and non-consumption groups was 157.3 mg/day and 166.2 mg/day, respectively, lower than the taurine content of the average normal adults in 1998, which was 177 mg/day for adults residing in Seoul. The present finding that the average taurine intake in the consumption group was less than that of the non-consumption group is in agreement with a previous study (Park et al. 2001).

Among males, the average intake of Ca, Vit B_2 in the consumption and non-consumption groups was 460.6 mg/day, 1.2 mg/day and 499.3 mg/day, 1.2 mg/day, respectively. Among females, the average intake of Ca in the consumption and non-consumption groups was 471.5 mg/day and 395.9 mg/day, respectively, while the average intake of Vit B_2 in the consumption and non-consumption groups was 1.1 mg/day and 1.0 mg/day, respectively. Significant differences in dietary Ca and Vit B_2 intake was observed between the two groups ($p<0.05$) in females.

Table 3 Dietary taurine and nutrient intakes by HFF consumption in males

Variables	Consumer (n=65)	Non-consumer (n=97)	t-value
Taurine (mg/day)	139.2±7.7[a]	171.0±8.2	–2.633**[b]
Energy (kcal/day)	1905.2±46.2	1961.3±40.1	–0.905
Carbohydrate (g/day)	251.3±6.8	256.1±5.8	–0.533
Total protein (g/day)	80.3±3.5	81.9±2.5	–0.371
Total fat (g/day)	62.3±2.2	64.9±2.1	–0.843
Total Ca (mg/day)	460.6±20.1	499.3±20.9	–1.272
Total Fe (mg/day)	12.9±0.6	13.1±0.4	–0.376
Vit A (μgRE/day)	676.1±30.3	700.5±32.9	–0.517
Vit B_1 (mg/day)	1.5±0.05	1.4±0.04	0.822
Vit B_2 (mg/day)	1.2±0.04	1.2±0.03	–0.344
Vit C (mg/day)	77.0±5.0	75.3±5.8	0.203
Folic acid (mg/day)	209.4±8.4	204.5±6.8	0.456

[a]Means±SE

[b]Superscripts are significantly different between HFF consumption by Student *t*-test (**p<0.01)

Table 4 Dietary taurine and nutrient intakes by HFF consumption in females

Variables	Consumer (n=43)	Non-consumer (n=46)	*t*-value
Taurine (mg/day)	157.3±15.9[a]	166.2±13.2	–0.432
Energy (kcal/day)	1662.4±44.2	1648.9±46.0	0.211
Carbohydrate (g/day)	224.9±6.8	221.8±6.1	0.318
Total protein (g/day)	65.8±2.4	68.3±3.6	–0.569
Total fat (g/day)	53.0±2.3	55.7±2.7	–0.731
Total Ca (mg/day)	471.5±22.9	395.9±18.0	2.612*[b]
Total Fe (mg/day)	11.5±0.4	11.2±0.5	0.509
Vit A (μgRE/day)	674.3±31.7	659.9±37.1	0.292
Vit B_1 (mg/day)	1.1±0.05	1.1±0.05	–0.225
Vit B_2 (mg/day)	1.1±0.05	1.0±0.04	2.430*
Vit C (mg/day)	69.2±5.1	63.3±4.9	0.834
Folic acid (mg/day)	198.6±9.8	179.6±8.2	1.475

[a]Means±SE

[b]Superscripts are significantly different between HFF consumption by Student *t*-test (*p<0.05)

4 Conclusion

In this study, most subjects preferred HFF for control of fat, to purchase their HFF in a specialty store and to consult a specialized consultant on HFF.

Therefore, for Korean college students, development of health functional taurine-containing food for anti-obesity activity is needed, and HFF should be sold in specialty stores, which provide a specialized consultant.

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Taurine Accelerates Alcohol and Fat Metabolism of Rats with Alcoholic Fatty Liver Disease

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Abbreviations

AFLD	Alcoholic fatty liver disease
AG	Triglycerides
ALD	Alcoholic liver disease
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
HDL-C	High density lipoprotein cholesterol
LDL-C	Low density lipoprotein cholesterol
NEFA	Non-esterified fatty acid
TC	Total cholesterol
TG	Triglyceride

1 Introduction

Alcohol related liver disease (ARLD), a condition in which alcohol overuse causes liver damage, consists of four stages. Alcoholic fatty liver disease (AFLD) which is the first stage of ARLD, is characterized by abnormal accumulation of triglycerides (AG) within parenchymal cells. It is asymptomatic, reversible and the most common of the four stages of ARLD, the other three being alcoholic hepatitis (AH), alcoholic hepatic fibrosis (AF) and alcoholic cirrhosis (AC). Alcohol is mainly metabolized in the liver, which is also the main organ responsible for lipid

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metabolism. Over consumption of alcohol will significantly damage hepatic cells as well as alter lipid metabolism.

Taurine, 2-aminoethanesulfonic acid, is an amino acid widely distributed in animal and human tissues. It has many fundamental biological functions, including conjugation of bile acids, antioxidation, osmoregulation, membrane stabilization and modulation of calcium signaling. Dietary supplementation with taurine was found effective in treating NAFLD (Gentile et al. 2011). In iron-potentiated alcoholic liver fibrotic rats, taurine restored mitochondrial function, reduced reactive oxygen species formation, prevented DNA damage and apoptosis, curtailed the production of inflammatory and fibrogenic mediators and the activation of stellate cells (Lakshmi Devi and Anuradha 2010; Devi et al. 2010). Our previous study also found that taurine prevents and cures alcoholic liver disease in rats by partially preventing oxidative damage and inflammation caused by alcohol (Wu et al. 2009); taurine alone as well as coadministration with Chinese traditional medicine accelerates alcohol metabolism in rats with acute alcohol administration (Wu et al. 2013).

In this study, the effects of taurine on alcohol and lipid metabolism in AFLD rats were investigated in order to provide information on potential AFLD therapy.

2 Materials and Methods

2.1 Animals and Groups

Male Wistar rats weighing 100–140 g were acclimatized to laboratory conditions for 1 week before starting the experiment. They were maintained under standard conditions of temperature (23 ± 2 °C) and humidity (40 %) with alternating 12 h light/dark cycles. The rats had free access to standard diet and water ad libitum. The whole experiment included preventive trials and curative trials. In preventive trials, rats were distributed into six groups: rats in the model group (M) were intragastrically administered 40 % alcohol (15 ml/kg/day) and pyrazole (24 mg/kg/day, Sigma Chemical Company, St. Louis, MO, USA) everyday for 12 weeks and were fed a high-fat diet (commercial standard rat chow dipped in corn oil) in order to establish the AFLD models. Rats in the taurine preventive groups (PI, PII) received the same treatment as rats in the M group except for drinking water, which contained either 2 % (PI) or 5 % (PII) taurine (Jeaky technology development company Ltd, Beijing, China). Rats in the normal group (N) were intragastrically administered daily with the same volume of saline and given standard diet and tap water. Rats in the control groups (CI, CII) received the same treatment as rats in the N group except for drinking water, which contained either 2 % (CI) or 5 % (CII) taurine. In the curative trials, the normal group (N) were given standard food and water; the AFLD model rats were divided into three groups: the automatic recovery group (A) were given standard food and water, rats in the taurine treatment groups (TI, TII) were given water containing either 2 % (TI) or 5 % (TII) taurine for 4 and 8 weeks. At the end of the experiment, the rats were euthanized and then blood and liver were collected for biochemical analysis.

2.2 Biochemical Analysis

Blood samples were collected from the jugular vein. After standing at room temperature for 45 min, serum was separated by centrifuging at 1,500 rpm for 15 min at 4 °C. Liver tissues were taken and rapidly homogenized in ice-cold saline. Tissue homogenates were centrifuged at 3,000 rpm for 10 min at 4 °C to remove crude matter. Then the supernatants were collected. Serum alanine aminotransferase (ALT), aspartate aminotransfer (AST), serum and hepatic non-esterified fatty acid (NEFA), and hepatic activities of ADH (alcohol dehydrogenase) and ALDH (acetaldehyde dehydrogenase) were measured by colorimetry using kits according to instructions from the manufacturer (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Serum total cholesterol (TC), triglyceride (TG), high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C) in rat serum were measured at 500 nm, 500 nm, 500 nm and 520 nm, using kits according to the instructions from the manufacturer (Beijing Beihua kangtai clinical agent company Ltd, Beijing, China).

2.3 Histological Analysis

Hepatic tissues from the left hepatic lobe were fixed in 10 % (v/v) phosphate buffered formalin solution (pH 7.0) and embedded in paraffin wax. Sections were cut into 5 μm slices and stained with haematoxylin and eosin. The adipohepatic percentage was the ratio of the adipohepatic area to the area of the total visual field. The degrees of hepatic steatosis were classified according to Table 1.

2.4 Statistical Analysis

Results were presented as means ± SEM. The statistical analyses were carried out using one-way analysis of variance (ANOVA) followed by a post hoc DUNCAN's multiple range test or Tukey's post hoc test. A statistically significant difference is defined as a p-value of less than 0.05.

Table 1 Classification of the degrees of hepatic steatosis

Degrees	Steatosis percentage (%)
1	≤1
2	1–2
3	2–5
4	5–10
5	10–20
6	>20

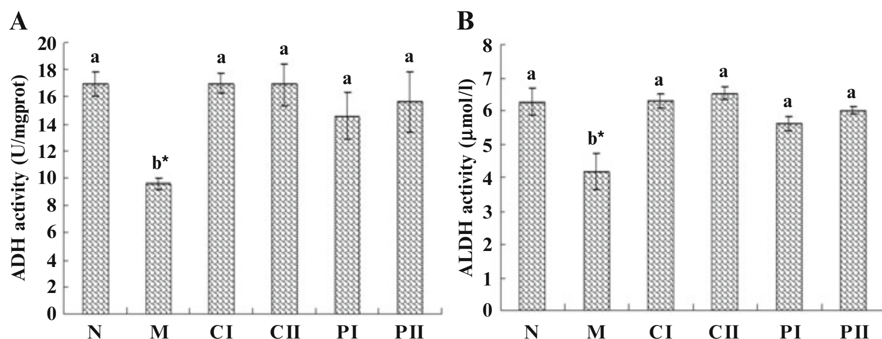

Fig. 1 (**a**) Effects of taurine on hepatic activities of ADH in rats. (**b**) Effects of taurine on hepatic activities of ALDH in rats. Rats in the model group (M) were intragastrically administered daily with 40 % alcohol (15 ml/kg/day) and pyrazole (24 mg/kg/day) for 12 weeks and were fed a high-fat diet. Rats in the taurine preventive groups (PI, PII) received the same treatment as rats in the M group except for drinking water, which contained either 2 or 5 % taurine. Rats in the normal group (N) were intragastrically administered daily the same volume of saline and provided standard diet and tap water. Rats in the control groups (CI, CII) received the same treatment as the rats in the N group except for drinking water, which contained either 2 or 5 % taurine. Each column and vertical bar represents the means ± SEM. In comparison to the normal group, letter superscripts ($P < 0.05$) and asterisks ($P < 0.01$) denote significant differences from the normal group (ANOVA followed by the Tukey's post hoc test)

3 Results

3.1 Effects of Taurine on Alcohol Metabolism in the Liver of Rats

As seen in Fig. 1, compared with the normal group, hepatic activities of ADH and ALDH in model group after 12 weeks were significant lower ($P < 0.05$); the model group also showed significant differences compared with taurine groups ($P < 0.05$). However, there were no obvious differences between the normal group and the taurine groups ($P > 0.05$).

3.2 Effects of Taurine on Steatosis of Liver in AFLD Rats

The percentage of diseased liver cells was calculated from the extent of steatosis observed by microscopy. The number of adipohepatic cells in the liver of AFLD rats was much larger than those of the normal rats ($P < 0.01$); there were no significant differences between 2 %, 5 % taurine control and the normal groups ($p > 0.05$). The degree of steatosis was less in the 2 % taurine preventive group compared with the model group, while there were significant differences between the model group and

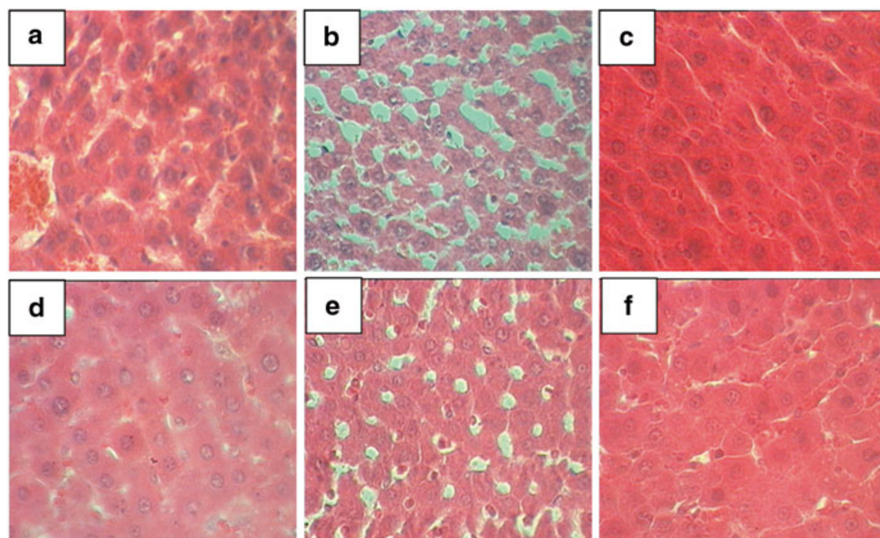

Fig. 2 Effect of preventive administration of taurine on steatosis in the liver. (**a**) In the normal group (N), rats were intragastrically administered saline daily and were fed with standard diet and tap water; (**b**) Rats in the model group (M) were intragastrically administered 40 % alcohol (15 ml/kg/day) and pyrazole (24 mg/kg/day) for 12 weeks and fed a high-fat diet; (**c**) In control group I (CI), rats received the same treatment as the rats in N group except for drinking water, which contained 2 % taurine; (**d**) Rats in control group II (CII) received the same treatment as rats in N group except for the drinking water, which contained 5 % taurine; (**e**) The 2 % taurine preventive group (PI) received the same treatment as rats in the M group except for drinking water, which contained 2 % taurine; (**f**) Rats in the 5 % taurine preventive group (PII) received the same treatment as rats in M group except for drinking water, which contained 5 % taurine. The liver was collected after 12 weeks and fixed in 10 % phosphate buffered formalin solution and embedded in paraffin wax. Sections were cut into 7 µm slices and stained with haematoxylin and eosin (H.E)

Table 2 Preventive administration of taurine on hepatic pathologic changes

Group	Degrees	Steatosis percentage
N	1	0.62 ± 0.26^{a}
M	6	22.02 ± 2.33^{b}
CI	1	0.73 ± 0.21^{a}
CII	1	0.78 ± 0.08^{a}
PI	4	5.17 ± 0.11^{c}
PII	2	1.01 ± 0.16^{a}

Results represent means ± SE (n = 5). Rats in the model group (M), CI group, CII group, PI group and PII group were treated as described in Fig. 2. Each superscript letter (a–c) denotes a significant difference with values associated with a different letter ($P < 0.05$)

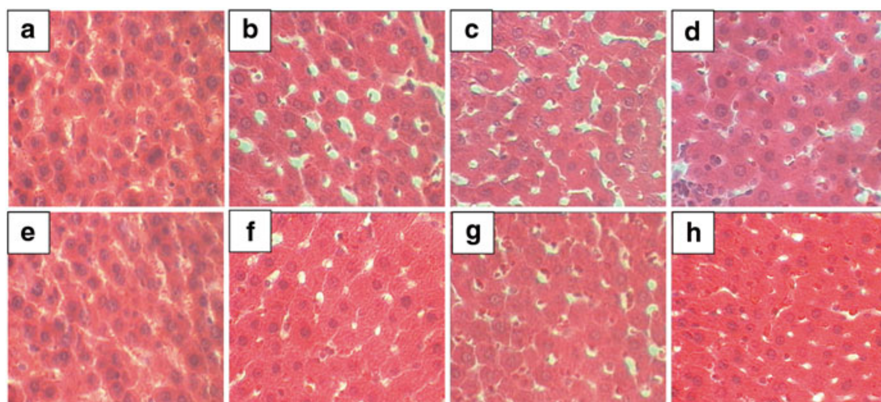

Fig. 3 Curative administration of taurine on hepatic steatosis. (**a**) Normal (N) rats were given standard food and water for 4 weeks; (**b**) The automatic recovery group (A) consist of AFLD rats given standard food and water for 4 weeks; (**c**) The taurine treatment group I (TI) are AFLD rats given water containing 2 % taurine for 4 weeks; (**d**) The taurine treatment group II (TII) are AFLD rats given water containing 5 % taurine for 4 weeks. (**e**) Normal (N), rats were given standard food and water for 8 weeks; (**f**) The automatic recovery group (A) consist of AFLD rats given standard food and water for 8 weeks; (**g**) The taurine treatment group I (TI) represent AFLD rats given water containing 2 % taurine for 8 weeks; (**h**) The taurine treatment group II (TII) are AFLD rats given water containing 5 % taurine for 8 weeks. Liver was collected after the experiment and fixed in 10 % phosphate buffered formalin solution before being embedded in paraffin wax. Sections were cut into 7 μm slices and stained with H.E

Table 3 Curative administration of taurine on hepatic steatosis

	4 weeks		8 weeks	
Group	Degree	Steatosis percentage	Degree	Steatosis percentage
N	1	0.47 ± 0.15^{a}	1	0.54 ± 0.20^{a}
A	4	5.61 ± 0.61^{b}	3	3.51 ± 0.57^{b}
TI	3	3.14 ± 0.44^{c}	2	1.95 ± 0.37^{c}
TII	3	2.50 ± 0.59^{c}	2	1.67 ± 0.60^{ac}

Results represent means ± SE (n = 5). Rats were treated as described in Fig. 3. Values with different superscript letters (a–c) in the same row represent significant differences ($p < 0.05$); no significant difference exists between values containing the same letter ($p > 0.05$)

the 5 % taurine preventive group ($P < 0.01$), the latter which was similar to the normal rats.

After 4 weeks of treatment, fat vacuoles could be seen in the automatic recovery group; the degree of adipose degeneration lessened in the 2 and 5 % taurine treatment groups ($P < 0.05$), but there were still significant differences compared to the normal rats. After 8 weeks treatment, rats in the automatic recovery group showed significant adipose degeneration compared to the normal group ($P < 0.05$), but there were no significant differences between the 5 % taurine and the normal groups ($P > 0.05$).

3.3 Effects of Taurine on Serum ALT and AST

Serum ALT and AST of the preventive groups increased significantly compared with the normal rats ($P < 0.05$), while there were no obvious differences between the taurine control and the normal groups ($P > 0.05$). Serum ALT and AST in both the 2 and 5 % taurine preventive groups decreased significantly compared with the untreated AFLD rats ($P < 0.05$).

In the curative trial after the end of the fourth week, serum ALT and AST in the automatic recovery group were significant higher than the normal group ($P < 0.05$),

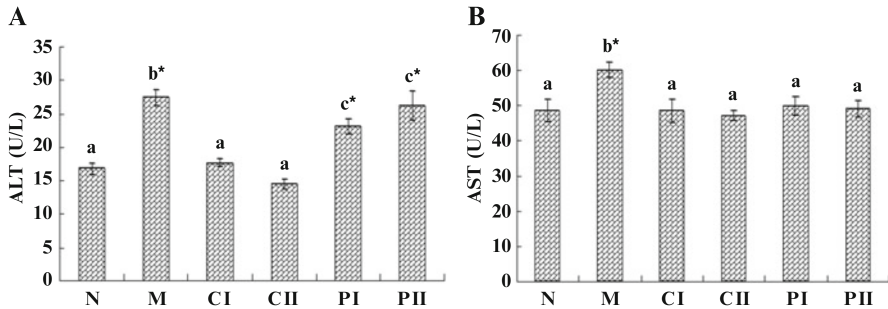

Fig. 4 Preventive administration of taurine on the activities of serum ALT and AST of AFLD rats. The groups of rats are identical to those described in Fig. 2. Each *column* and *vertical bar* represents means ± SEM. Significant differences with respect to the normal group are denoted by *letter superscripts* (*a–c*) ($P < 0.05$) and *asterisks* ($P < 0.01$)

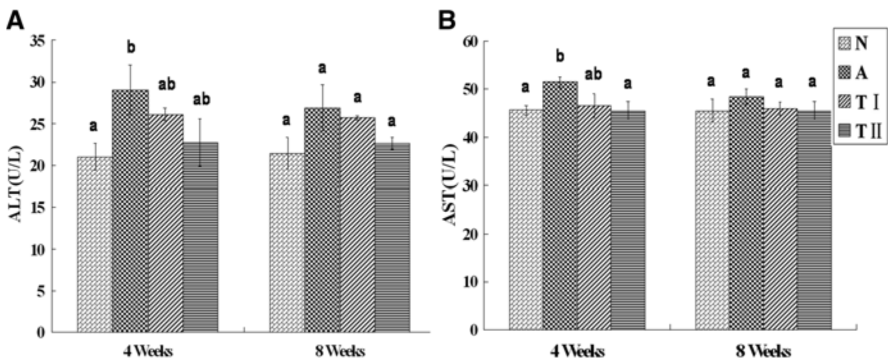

Fig. 5 Curative administration of taurine on the activities of serum ALT and AST. Rats in the normal group (N) were given standard food and water. Rats in the automatic recovery group (A) were given standard food and water after establishing the AFLD model. Rats in the taurine treatment group I (TI) were given water containing 2 % taurine for 4 weeks and 8 weeks after model establishment. Rats in taurine treatment group II (TII) were given water containing 5 % taurine for 4 weeks and 8 weeks after model establishment. Each *column* and *vertical bar* represent the mean ± SEM. *Superscript letters* and *asterisks* represent significant differences ($p < 0.05$ and $p < 0.01$, respectively) between the experimental AFLD groups and the normal group

while the taurine treatment groups showed no significant difference (p>0.05) compared with the normal group. At the end of the eighth week, there were no significant differences among all the groups (P>0.05).

3.4 *Effects of Taurine on Lipid Metabolism Indexes of AFLD Rats*

In the preventive trial, serum TC, TG and LDL-C in the AFLD group increased significantly compared with the normal group (P<0.05). The levels in the taurine preventive groups were all significantly lower than the model rats except for serum TC in the 2 % taurine preventive group, which exhibited no obvious difference compared with the model group. Serum levels of HDL-C in the model group were significantly reduced compared with those of the normal group, while serum HDL-C in the taurine preventive groups increased compared with the model group (P<0.05). All indexes in the taurine control groups were not significantly different from the normal group (P>0.05).

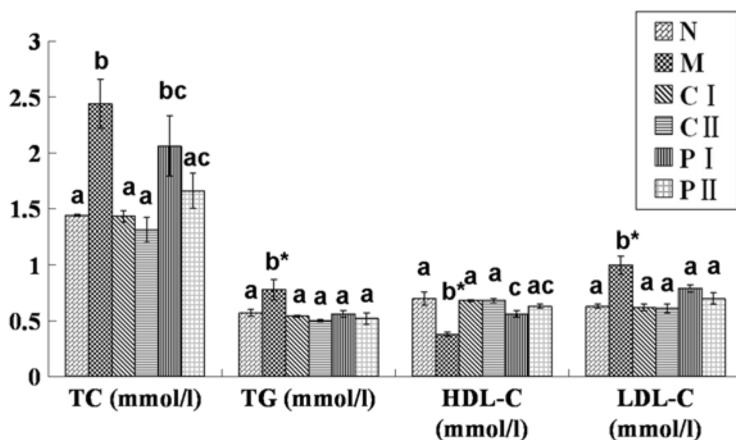

Fig. 6 Preventive administration of taurine on the concentrations of serum TC, TG, HDL-C and LDL-C in rats. Rats in the model group (M) were intragastrically administered 40 % alcohol (15 ml/kg/day) and pyrazole (24 mg/kg/day) daily for 12 weeks and fed a high-fat diet. Rats in the taurine preventive groups (PI, PII) received the same treatment as rats in the M group except the drinking water contained either 2 % (PI) or 5 % taurine (PII). Rats in the normal group (N) were intragastrically administered the same volume of saline daily and provided standard diet and tap water. Rats in the control groups (CI, CII) received the same treatment as the rats in the N group except the drinking water contained either 2 % (CI) or 5 % (CII) taurine. Each column and vertical bar represents the mean ± SEM. *Superscript letters* and *asterisks* denote significant differences (P<0.05 and p<0.01, respectively) from the normal group

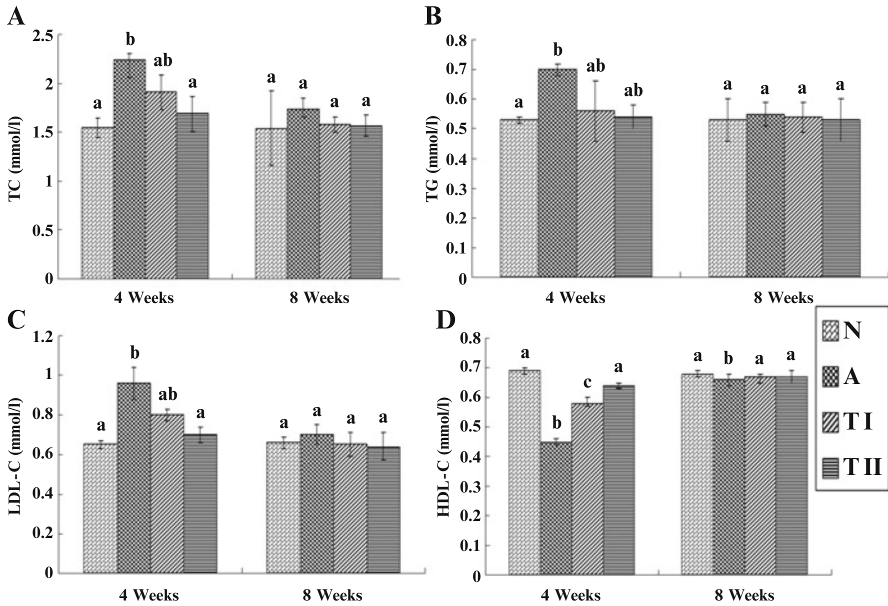

Fig. 7 Curative administration of taurine on the concentrations of serum TC, TG LDL-C and HDL-C. Rats in the normal group (N) were provided standard food and water. Rats in the automatic recovery group (A) were given standard food and water after the AFLD model was established. Rats in the taurine treatment group I (TI) were given water containing 2 % taurine for 4 weeks and 8 weeks after model establishment. Rats in the taurine treatment group II (TII) were given water containing 5 % taurine for 4 weeks and 8 weeks after model establishment. Each *column* and *vertical bar* represents the mean ± SEM. *Superscript letters* and *asterisks* denote significant differences ($P<0.05$ and $P<0.01$, respectively) compared with the normal group

Compared with the automatic recovery group, 4 weeks of taurine treatment lowered serum TC, TG, LDL-C to values virtually identical to that of the normal group. Serum HDL-C in the automatic recovery groups was lower than that of the normal rats ($P<0.05$), while the 5 % curative group was statistically identical to that of the normal group ($P>0.05$). At the end of the eighth week of taurine treatment, serum TC, TG, LDL-C, HDL-C were indistinguishable among the four groups ($P>0.05$), except for serum HDL-C in the automatic recovery group which remained elevated relative to that of the normal group ($P<0.05$).

Serum and hepatic levels of NEFA in the AFLD model group increased significantly compared with the normal group ($P<0.05$). The levels of NEFA in the taurine preventive groups decreased compared with the model rats ($P<0.05$). There were no significant differences between the taurine control and the normal groups.

At the fourth weekend of the curative trial, hepatic and serum NEFA in the automatic recovery group were significantly higher ($P<0.01$), while the taurine curative groups were not significantly different from the normal group ($P>0.05$). After the eighth week of the curative trial, serum NEFA in the automatic recovery group was higher than the normal and taurine curative groups ($P<0.05$), while the taurine curative groups exhibited no difference from the normal rats ($P>0.05$).

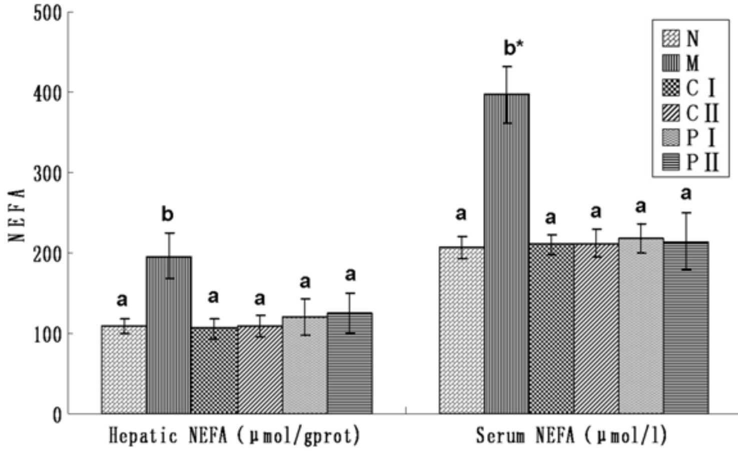

Fig. 8 Preventive administration of taurine on serum and hepatic concentrations of NEFA. Rats in the model group (M) were intragastrically administered 40 % alcohol (15 ml/kg/day) and pyrazole (24 mg/kg/day) daily for 12 weeks and fed a high-fat diet. Rats in the taurine preventive groups (PI, PII) received the same treatment as rats in the M group except for drinking water, which contained either 2 % (PI) or 5 % (PII) taurine. Rats in the normal group (N) were intragastrically administered the same volume of saline daily and given standard diet and tap water. Rats in the control groups (CI, CII) received the same treatment as the rats in the N group except for drinking water, which contained either 2 % (CI) or 5 % (CII) taurine. Each *column* and *vertical bar* represents the mean ± SEM. *Superscripts* [*letters* ($p<0.05$) and *asterisks* ($p<0.01$)] signify significant differences compared with the normal group

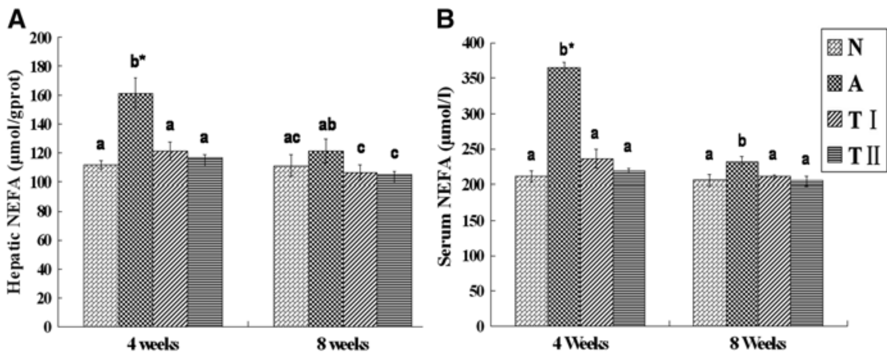

Fig. 9 Curative administration of taurine on hepatic and serum concentrations of NEFA. Rats in the normal group (N) were provided standard food and water. Rats in the automatic recovery group (A) were given standard food and water after establishment of the model. AFLD rats in the taurine treatment group I (TI) were given water containing 2 % taurine for 4 and 8 weeks. Rats in the taurine treatment group II (TII) were given water containing 5 % taurine for 4 and 8 weeks. Each *column* and *vertical bar* represents the mean ± SEM. *Superscript letters* ($p<0.05$) and *asterisks* ($p<0.01$) denote significant differences compared with the normal group

4 Discussion

Alcohol is mainly metabolized into acetaldehyde by ADH in the liver, which in turn is oxidized to acetic acid by ALDH, with the end product of this process being CO_2 and H_2O_2 at a concentration that is harmless to the liver. However, overconsumption of alcohol lowers the activities of ADH and ALDH, leading to the accumulation of alcohol and its harmful metabolic products in the liver, thereby causing serious hepatotoxicity. In this experiment, the activities of ADH and ALDH decreased significantly in AFLD rats. Thus, the metabolism of alcohol is inhibited, causing harmful metabolic products to accumulate in the liver inducing injury of hepatic cells. Because taurine administered preventively to rats fed alcohol significantly increased the activities of ADH and ALDH, the metabolism of alcohol and acetaldehyde is promoted and the damage caused by alcohol and its metabolic products is reduced in the hepatic cells.

AFLD damages hepatic cells, changing the permeability of the cell membrane or even leading to cellular swelling and disruption. AST and ALT, which are two main aminotransferases inside the hepatic cells under normal conditions, are released from damaged hepatocytes into the blood. Their concentrations are the main sensitive indexes of liver damage. It has been reported that AST increases significantly after alcoholic liver damage, but ALT is not positively correlated with the degree of hepatic damage (Ohkawa et al. 1979). This conclusion is not in accordance with this experiment. Our results showed that both serum ALT and AST increased significantly in AFLD model rats, which indicates that serum ALT and AST are both sensitive indices of liver damage, while taurine administered preventively inhibits this increase; 4 weeks after taurine treatment, serum AST and ALT in AFLD rats decrease to levels seen in normal rats. Eight weeks after taurine treatment, serum ALT and AST nearly recover completely. The results indicate that the hepatic cell membrane can automatically repair after alcohol withdrawal if the damage is not serious, while taurine accelerates the repair of hepatic cells, decreasing membrane permeability and inhibiting the release of ALT and AST.

Liver is also the main organ responsible for lipid metabolism. Hepatic cell injury leads to abnormal lipid metabolism in the liver. Under this situation, lipids that are not fully oxidized accumulate inside the hepatic cell. Under physical conditions, part of the serum NEFA absorbed by hepatic cells are oxidized in the chondriosome to provide energy, the other part of NEFA is converted to phosphatide, TC and TG in the smooth endoplasmic reticulum. Fat synthesis decreases when the NEFA are fully oxidized; otherwise, the synthesis of TC and TG increases before being released and transported into the blood. The increase in serum TC and TG implies an over-synthesis and accumulation of TC and TG in hepatic cells. This pathological condition has been named AFLD, which is characterized by adipose degeneration and lipid droplet accumulation in hepatic cells (Ruijie Liu 1999). Cholesterol transportation in the blood requires apolipoprotein. HDL, mainly synthesized in the liver, extracts exogenous cholesterol from the membrane of cells, and then converts into HDL-C, which can be taken up by the liver. On the other side, LDL is responsible for transporting endogenous cholesterol synthesized inside the liver to the

other tissues. The increase LDL-C and the decrease HDL-C in the serum is indicative of large amounts of cholesterol being transported into the liver and the accumulation of TC in the liver. Elevations of LDL-C in the serum may also signify increases in cholesterol synthesis in the liver.

Taurine, which is mainly synthesized by the liver, regulates the synthesis of bile acids and the concentration of free cholesterol in vitro (Yokogoshi et al. 1999). In vivo, taurine administered in rat food inhibits the rise in cholesterol (Yan Chongchao 1988). Addition of taurine to the water of hypercholesterolemia rats causes an increase in serum HCL-C but a decrease in serum TC (Tianpei He and Jingfan Gu 1997). A recent report on chronic alcoholic patients has found that taurine decreases AST and ALT activities and the levels of cholesterol, triglyceride (TG) of serum and plasma, while increasing ADH and ALDH activities (Hsieh et al. 2014). These results are in accordance with the present study showing that serum TC, TG, LDL-C, NEFA and hepatic NEFA of AFLD rats significantly increase while serum HDL-C significantly decreases. Taurine administered preventively and curatively lower serum TC, TG, LDL-C, NEFA and hepatic NEFA, while raising serum levels of HLD-C. The results indicate that taurine accelerates the transportation of cholesterol out of the hepatocyte, as well as transform cholesterol inside the hepatocyte into bile acids, the latter resulting in a decrease in serum TC content. What's more, taurine accelerates β oxidation by regulating enzymes. Thus, most NEFA absorbed by the liver becomes directly oxidized to provide energy. By this mechanism taurine reduces the synthesis of TG in the liver. Meanwhile, taurine also increases the decomposition of TG, reduces the deposition of TG in the liver and lowers serum TG concentration.

5 Conclusion

Taurine significantly inhibits and reverses hepatic injury caused by alcohol through the stimulation of alcohol metabolism by ADH and ALDH. Hence, taurine administered preventively and curatively accelerates lipid metabolism in the liver of AFLD rats.

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Protective Effect of Mussel (*Mytilus Coruscus*) Extract Containing Taurine Against AAPH-Induced Oxidative Stress in Zebrafish Model

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Abbreviations

AAPH	2,2′-Azobis dihydrochloride
DPPH	1,1-Diphenyl-2-picrylhydrazyl
ESR	Electron spin resonance

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1 Introduction

Taurine (2-aminoethane sulfonic acid) is synthesized from methionine via cysteine by a series of enzymatic reactions and has been reported to be present in high amounts in marine animals, mammalian tissues and blood cells (Allen and Garret 1972). Several researchers have demonstrated that taurine possesses several biological functions, including osmotic regulation, cell membrane stabilization and immune defense enhancement and antioxidant protection (Redmond et al. 1998; Han et al. 2000; Métayer et al. 2008).

In general, oxidative damage caused by reactive oxygen species (ROS) is accepted to play a role in age-related changes in tissues. Excess ROS formation can damage cellular macromolecules, such as lipids, proteins and DNA, and can cause cell damage (Kregel and Zhang 2007). ROS is also believed to play an important role in the occurrence of diseases, such as cardiovascular diseases, diabetes mellitus and neurological disorders. Therefore, it is important to inhibit the formation of free radicals and the oxidation occurring in the living body (Stadtman 2006; Peng et al. 2009).

Marine organisms, in particular, exhibit high levels of taurine, as it is the main organic osmolite in marine species. Raw mussel is the main source of taurine, with typical levels of 655 mg per 100 g, followed by fresh clams and raw white fish flesh with 240 mg and 151 mg per 100 g, respectively (Omura and Inagaki 2000). The antioxidant defense system of marine mussels has been discussed relative to pollution and heavy-metal toxicity (Manduzio et al. 2004). On the other hand, taurine deficiency has been implicated in respiratory chain dysfunction that allows accumulation of electron donors which divert electrons from the respiratory chain to oxygen, forming superoxide anion in the process (Schaffer et al. 2009). Although a number of studies concerning the effect of taurine on oxidative stress have been reported, the antioxidative effect of taurine in zebrafish model has not been validated. Therefore, the present study was conducted to investigate the potential protective efficacy of mussel water extract possessing high concentration of taurine on AAPH-induced oxidative stress in the zebrafish model.

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2 Methods

2.1 Reagents

5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO), 2,2-azobis (2-amidinopropane) hydrochloride (AAPH), 1,1-diphenyl-2-picrylhydrazyl (DPPH) and *alpha*-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (4-POBN) were obtained from Sigma Chemical Co. (St. Louis, MO, USA), All other reagents were of the highest grade available commercially.

2.2 Measurement of Free Radical Scavenging Activity

2.2.1 DPPH Radical Scavenging Activity

DPPH radical scavenging activity was measured using the method described by Nanjo et al. (1996). Briefly, 60 μL of mussel extract at each concentration was added to 60 μL of DPPH (60 μM) in a methanol solution. After mixing vigorously for 10 s, the solution was transferred into a 100 μL Teflon capillary tube, and the scavenging activity of each sample for the DPPH radical was measured using an ESR spectrometer. A spin adduct was measured with an ESR spectrometer exactly 2 min later. Experimental conditions were as follows: central field, 3,475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 5 mW; gain, 6.3×10^5 and temperature, 298 K.

2.2.2 Alkyl Radical Scavenging Activity

Alkyl radicals were generated by AAPH. The phosphate-buffered saline (pH 7.4) reaction mixtures containing 10 mM AAPH, 10 mM 4-POBN, and the indicated concentrations of tested samples were incubated at 37 °C in a water bath for 30 min and then transferred to a 100 μL quartz capillary tube. The spin adduct was recorded using an ESR spectrometer. Measurement conditions were as follows: central field, 3,475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 1 mW; gain, 6.3×10^5; and temperature, 298 K.

2.3 Chemical Analysis and Amino Acid Composition

The mussel sample was analyzed for crude protein, crude fat (ether extract) and total ash content using the methods of the Association of Official Analytical Chemists (AOAC 1990). For amino acid analysis, mussel samples (80 mg) were combined with 10 mL of 6 N HCl. After N_2 gas was used to purge the samples in

the test tube, the samples were hydrolysed in a dry oven at 110 °C for 24 h. The hydrolysed samples were then evaporated and a sodium-distilled buffer (pH 2.2) was added. Samples were then filtered through a syringe filter (0.45 μm) and analyzed using an amino acid autoanalyzer (Pharmacia Biotech Biochrom 20, Ninhydrin Method). Amino acids were determined by absorbance at 440 and 570 nm.

2.4 In Vivo *Zebrafish Model*

2.4.1 Origin and Maintenance of Parental Zebrafish

Adult zebrafish were obtained from a commercial dealer (Seoul aquarium, Seoul, Korea) and 15 fish were kept in 3.5 L acrylic tank with the following conditions; 28.5 ± 1 °C, and were fed twice times a day (Tetra GmgH D-49304 Melle Made in Germany) with a 14/10 h light/dark cycle. The day before the experiment, one female and two males interbred. In the morning (on set of light), embryos were obtained from the natural spawning collection of embryos were completed within 30 min into petri dishes containing media.

2.4.2 Waterborne Exposure of Embryos to AAPH

The embryos (n = 15) were transferred to individual wells of a 12-well plates containing 900 L embryo media from approximately 7 to 9 hpf. After 1 h incubation, a 15 mM AAPH solution was added to the embryo medium and incubated for up to 24 hpf. Then, embryos were rinsed using fresh embryo media.

2.4.3 Measurement of Intracellular ROS Generation

Generation of ROS production by zebrafish was analyzed using an oxidation-sensitive fluorescent probe dye, DCFH-DA. At 3 dpf, a zebrafish larva was transferred to one well of the 96-well plate, treated with DCFH-DA solution (20 g/mL) and incubated for 1 h in the dark at 28.5 ± 1 °C. After the incubation, the zebrafish larvae were rinsed by fresh embryo media and anaesthetized with 2-phenoxy ethanol (1/500 dilution sigma) before observation and photographed under the microscope CoolSNAP-Pro color digitalcamera (Olympus, Japan). A fluorescence intensity of individual larva was quantified using the image J program.

2.4.4 Measurement of Oxidative Stress-Induced Lipid Peroxidation Generation

Lipid peroxidation was measured to assess oxidative membrane damage in zebrafish. Diphenyl-l-pyrenylphosphine (DPPP) is fluorescent probe for detection of cell membrane lipid peroxidation. DPPP is non-fluorescent, but it becomes fluorescent

when oxidized. At 3 dpf, a zebrafish larva was transferred to one well of the 96-well plate, treated with DPPP solution (25 μg/mL) and then incubated for 1 h in the dark at 28.5 ± 1 °C. After the incubation, zebrafish larvae were rinsed using fresh embryo media and anaesthetized with 2-phenoxy ethanol (1/500 dilution sigma) before observation and photographed under the microscope CoolSNAP-Pro color digital camera (Olympus, Japan). A fluorescence intensity of individual zebrafish larva was quantified using the image J program.

2.4.5 Measurement of Oxidative Stress-Induced Cell Death

Cell death was detected in live embryos using acridine orange staining. Acridine orange stain cells with disturbed plasma membrane permeability, therefore, it preferentially stains necrotic or very late apoptotic cells. At 3 dpf, a zebrafish larva was transferred to one well of the 96-well plate, treated with acridine orange solution (7 g/mL) and incubated for 30 min in the dark at 28.5 ± 1 °C. After the incubation, the zebrafish larvae were rinsed with fresh embryo media and anaesthetized with 2-phenoxy ethanol (1/500 dilution sigma) before observation and photographed under the microscope CoolSNAP-Pro color digital camera (Olympus, Japan). Fluorescence intensity of individual zebrafish larva was quantified using the image J program.

2.5 Statistical Analysis

All data are presented as the means ± SEM. The data were evaluated by a one-way analysis of variance. Differences between the mean values were assessed using the Tukey-Kramer multiple comparison test. Statistical significance was considered for values of $P < 0.05$.

3 Results

3.1 Chemical Composition of Mussel (Mytilus coruscus)

The chemical composition of mussel is shown in Table 1. In the present study, the content of dry matter, crude protein, crude lipids and ash accounted for 98.59 %, 57.53 %, 8.41 % and 7.66 %, respectively.

3.2 Amino Acid Composition of Mussel (Mytilus coruscus)

The amino acid composition of mussel is shown in Table 2. Total amino acid concentration was markedly increased in the mussel water extract compared to the lysis buffer extract. In the present study, glycine and taurine were the most abundant

Table 1 Chemical composition of Mussel (*Mytilus coruscus*)

Chemical composition (%)	
Dry matter	98.59 ± 0.21
Crude protein	57.53 ± 0.14
Crude lipids	8.41 ± 0.26
Ash	7.66 ± 0.31

Results are mean values of three replicates ± SD. Expressed as % (g per 100 g) on a dry weight basis

Table 2 Amino acid composition of Mussel (*Mytilus coruscus*)

Amino acid composition (nmol/mg)	Water extract	Lysis buffer extract
Aspartic acid	21.82	9.43
Glutamic acid	41.82	10.26
Asparagine	6.66	0.95
Serine	25.42	6.55
Glutamine	19.97	3.67
Glycine	165.46	36.95
Histidine	4.97	2.01
Arginine	9.61	6.42
Taurine	140.76	33.72
Threonine	15.33	3.74
Alanine	82.53	20.58
Proline	19.03	4.68
Tyrosine	5.40	2.56
Valine	8.69	2.62
Methionine	2.97	0.58
Cysteine	1.08	0.00
Isoleucine	6.75	1.62
Leucine	9.00	2.45
Phenylalanine	3.37	1.25
Tryptophan	3.90	0.96
Lysine	4.89	2.45
Total	599.42	153.45

total free amino acids of mussel water extract. Taurine concentration of mussel water extract was 140.76 nmol/mg, which is 4.17-fold increased compared to that of lysis buffer extract. Based on these results, we evaluated the antioxidant activity of mussel water extract using the zebrafish model exposed to AAPH-induced oxidative stress.

3.3 *Radical Scavenging Activities of Mussel Extract*

In the present study, DPPH and alkyl radical scavenging activities of mussel extract were measured using an ESR spectrometer. The scavenging activity of mussel extract on DPPH and alkyl radical is shown in Fig. 1. The scavenging activity of

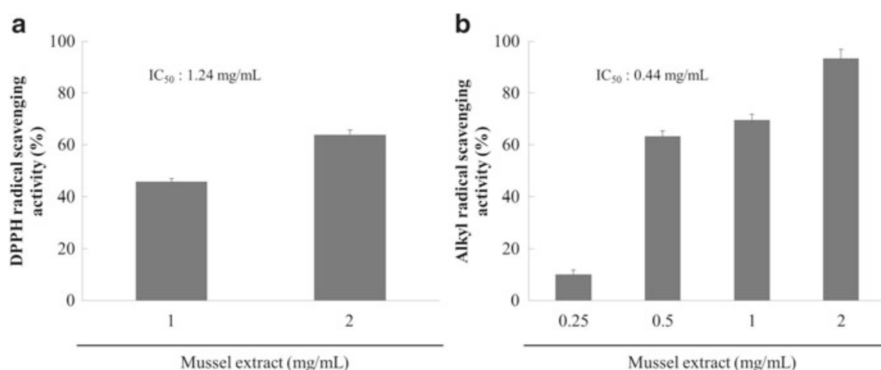

Fig. 1 Measurement of free radical scavenging activities of mussel extract by ESR. (**a**) DPPH scavenging activity and (**b**) alkyl radical scavenging activity. Values are means ± SD of triplicate experiments

mussel water extract against oxidative stress in zebrafish exposed to DPPH radical was expressed as the concentration that inhibited 50 % of free radical generation (IC_{50}), which in the case of the extract was 1.24 mg/mL (Fig. 1a). On the other hand, the mussel extract showed the greatest scavenging activity against alkyl radical (IC_{50} = 0.44 mg/mL) although it was also effective against DPPH radical (Fig. 1b).

3.4 *Measurement of Mussel Extract Against AAPH-Induced Cell Death* In Vivo *Zebra Fish Model*

Figure 2 shows that cell death is induced by AAPH treatment. The degree of cell death was dose-dependently and significantly reduced by adding mussel extract to AAPH-treated zebrafish ($P < 0.05$). When zebrafish were treated with mussel extract prior to AAPH treatment, a dramatic decrease in cell death was observed.

3.5 *Measurement of Mussel Extract Against AAPH-Induced Oxidative Stress and Lipid Peroxidation Generation* In Vivo *Zebra Fish Model*

We also determined the capacity of mussel extract to detect changes in the physiological state of zebrafish using DCFH-DA to assess the accumulation of ROS caused by the AAPH treatment. ROS converts DCFH-DA into non-fluorescent 2′,7′-dichlorodihydrofluorescein ($DCFH_2$) to the fluorescent 2′,7′-dichlorofluorescein (DCF); thus fluorescence intensity increases with ROS production (Walker et al. 2012). ROS levels were 156 % in AAPH-treated zebrafish compared to the control group. In contrast, zebrafish exposed to AAPH and mussel extract at different

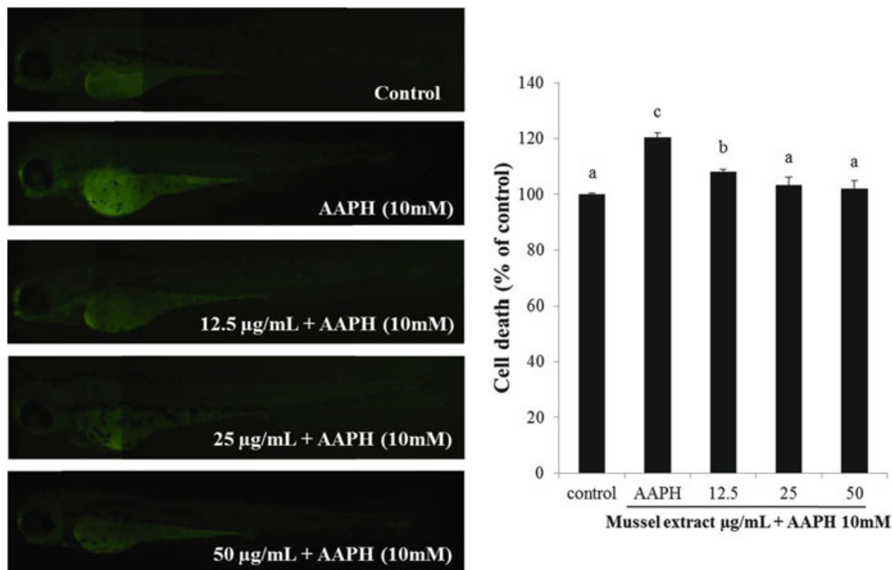

Fig. 2 Measurement of AAPH toxicity and mussel extract co-treatment on cell death. Experiments were performed in triplicate and data are means ± SEM. Values not sharing a *common letter* are significantly different at $P < 0.05$ by Tukey-Kramer multiple comparison test

concentrations (12.5, 25 and 50 µg/mL) showed a dose-dependent, significant reduction in ROS production (Fig. 3). This result suggests a reduction of ROS generation by mussel extract treatment.

Diphenylpyrenylphosphine (DPPP) is a probe that readily detects hydroperoxides and DPPP oxide (Akasaka et al. 1987). AAPH-induced lipid peroxidation is shown in Fig. 4. The AAPH-treated zebrafish revealed 127.5 % of lipid peroxidation, whereas, zebrafish groups treated with 12.5, 25 and 50 µg/mL mussel extract showed dramatically decreased lipid peroxidation to 107.8 %, 107.4 % and 104.7 %, respectively. This result indicates a reduction in lipid peroxidation by mussel extract treatment.

4 Discussion

Oxidative stress is a condition associated with an increased rate of cellular damage induced by oxygen and oxygen-derived oxidants commonly known as ROS. These ROS include oxygen free radicals, such as superoxide, hydroxyl, peroxyl and hydroperoxyl radicals, which cause tissue damage by a variety of mechanisms, including DNA damage, lipid peroxidation and protein oxidation (Sikka 1996).

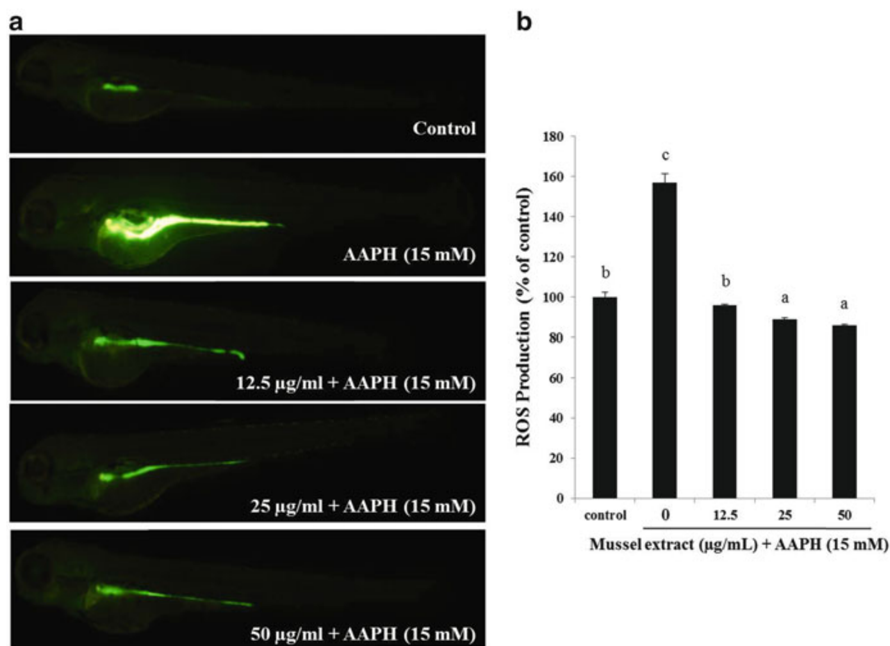

Fig. 3 Protective effect of mussel extract on AAPH-induced reactive oxygen species (ROS) production in zebrafish. (**a**) ROS levels were measured by image analysis and fluorescencemicroscope. (**b**) ROS levels were measured by Image J. Experiments were performed in triplicate and data are means ± SEM. Values not sharing a *common letter* are significantly different at $P < 0.05$ by Tukey-Kramer multiple comparison test

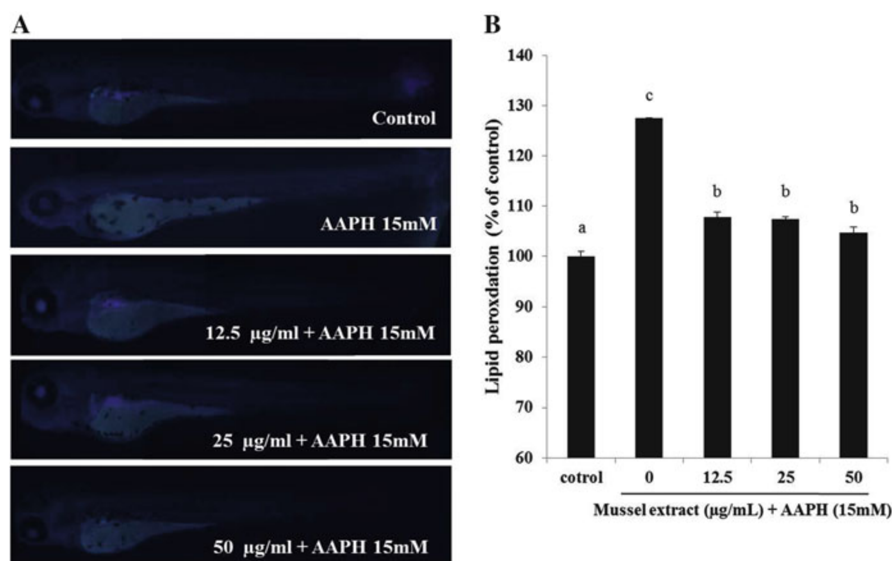

Fig. 4 Protective effect of mussel extract on AAPH-treated lipid peroxidation production in zebrafish. (**a**) Lipid peroxidation levels were measured by image analysis and fluorescence microscopy. (**b**) Lipid peroxidation levels were measured by Image J. Experiments were performed in triplicate and data are means ± SEM. Values not sharing a *common letter* are significantly different at $P < 0.05$ by the Tukey-Kramer multiple comparison test

In the present study, taurine was the most abundant total free amino acid of mussel water extract. Similarly, Beaulieu et al. (2013) reported that the nonessential amino acid group of aqueous phase from blue mussel was rich in taurine, aspartic acid, glutamic acid and glycine. Recently, taurine has become an attractive candidate for attenuating various toxin- and drug-induced pathophysiological conditions through its antioxidant action (Patrick 2006; Wang et al. 2008). Our data showed that mussel extract possessing high amounts of taurine has potent scavenging activities against DPPH and alkyl radical *in vitro*. The relatively stable DPPH radical has been widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors and thus to evaluate the antioxidant activity of various substances (Li et al. 2008).

In the present study, mussel water extract exhibited ROS scavenging and lipid peroxidation inhibiting effects. It also provided protection against AAPH-induced oxidative stress in zebrafish embryos. These results confirm that mussel extract have an ability to protect zebrafish embryos from oxidative stress-related cellular injuries. In this study, we also showed that mussel extract exhibits a prominent protective effect on AAPH-induced cell death. Similar with our results, Cozzi et al. (1995) reported that taurine has antioxidant activities by stabilizing biological membrane and scavenging reactive oxygen species. More recently, it has been reported that taurine treatment reversed several adverse effects and oxidative stress associated changes induced by endosulfan (Aly and Khafagy 2014). Also, several researchers demonstrated that taurine could be proposed to play its protective effect by its direct as well as indirect antioxidant activities. As a direct antioxidant activity, it could quench several reactive intermediates such as nitric oxide, hydrogen peroxide and hydroxyl radical (Cozzi et al. 1995). On the other hand, it could prevent the changes in oxidative stress-induced membrane permeability and stabilize it as an indirect antioxidant activity (Timbrell et al. 1995). Although a number of studies concerning the effect of taurine on antioxidant activities have been reported, no studies examined the protective effects of mussel water extract enriched in taurine on AAPH-induced oxidative stress in the zebrafish model.

Taken together, these results suggest that mussel extract possessing taurine has a beneficial effect on AAPH-induced oxidative stress *in vitro* as well as the *in vivo* zebrafish model by inhibiting intracellular ROS formation, lipid peroxidation and cell death.

5 Conclusion

Our present study shows that mussel water extract containing taurine exhibited DPPH and alkyl radical scavenging activity via ESR. In addition, we investigated for the first time the protective effects of mussel water extract against AAPH-induced oxidative stress in zebrafish embryos for the first time. Our results demonstrate that AAPH induces toxicity in zebrafish embryos and mussel extract can protect zebrafish embryos against AAPH, by inhibiting intracellular ROS formation, lipid peroxidation and cell death.

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Anti-inflammatory Effect of Short Neck Clam (*Tapes philippinarum*) Water Extract Containing Taurine in Zebrafish Model

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Abbreviations

LPS lipopolysaccharide
NO nitric oxide
ROS reactive oxygen species

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1 Introduction

Short neck clams (*Tapes philippinarum*) are a widely consumed shellfish in Asia. A number of *in vitro* and *in vivo* studies have found that these clams possess several medical and biological effects, including cholesterol-lowering, hepatoprotective and anti-tumorigenic properties (Prescott et al. 1974; Chijimatsu et al. 2009; Hsu et al. 2010). Current evidence demonstrates that freshwater clams have potent anti-inflammatory activity in rats (Huang et al. 2013).

Taurine is a sulfur-containing β-amino acid that is found in millimolar concentrations in most mammalian tissues and plasma as well as clams. It has been reported that the metabolic actions of taurine include bile acid conjugation, calcium homeostasis maintenance, glycolysis and glycogenesis stimulation (Hofmann and Small 1967; Kulakowski and Maturo 1984; Takahashi et al. 1992). Moreover, it has been shown in many *in vitro* and *in vivo* studies to have cytoprotective and immunomodulatory effects, and these actions are often attributed to an antioxidant mechanism (Schuller-Levis and Park 2004; Tappaz 2004). However, the metabolic actions of the water extract from short neck clam containing high concentration of taurine on inflammation using the zebrafish model have not yet been determined.

On the other hand, lipopolysaccharide (LPS) is a glycolipid component of the cell wall of gram-negative bacteria. It is known that the administration of LPS can stimulate the development of a systemic inflammatory response leading to nitric oxide (NO) synthesis (Bian and Murad 2001). Induction of NO synthesis during inflammation represents a defense mechanism against invading microorganisms, but excessive NO formation has been implicated in host tissue injury (Liu et al. 1993).

Recently, it has been reported that zebrafish have well-developed innate and acquired immune systems that are similar to that of the mammals (Trede et al. 2001). Therefore, the present study was designed to assess the anti-inflammatory effect of water extract from short neck clam containing taurine in the LPS-stimulated zebrafish model.

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2 Methods

2.1 Chemical Analysis and Amino Acid Composition

The short neck clam sample was analyzed for crude protein, crude fat (ether extract) and total ash content using the methods of the Association of Official Analytical Chemists (AOAC, 1990). For amino acid analysis, short neck clam samples (80 mg) were combined with 10 mL of 6 N HCl. After N_2 gas was used to purge the samples in the test tube, the samples were hydrolysed in a dry oven at 110 °C for 24 h. The hydrolysed samples were then evaporated and a sodium-distilled buffer (pH 2.2) was added. Samples were then filtered through a syringe filter (0.45 μm) and analyzed using an amino acid autoanalyzer (Pharmacia Biotech Biochrom 20, Ninhydrin Method). Amino acids were determined by absorbance at 440 and 570 nm.

2.2 Cell Culture

The cells of an African green monkey kidney (Vero) were maintained at 37 °C in an incubator, under a humidified atmosphere containing 5 % CO_2. The cells were cultured in DMEM containing 10 % heat-inactivated FBS, streptomycin (100 μg/mL), penicillin (100 unit mL^{-1}), and sodium pyruvate (110 mg L^{-1}).

2.3 Intracellular LPS Scavenging Assay by DCF-DA

For the detection of intracellular LPS (1 μg/mL), the Vero cells were seeded in 96-well plates at a concentration of 1.0×10^5 cells mL^{-1}. After 16 h, the cells were treated with samples (10 μL) and incubated at 37 °C under a humidified atmosphere. After 30 min, LPS was added at a concentration of 1 μg/mL, and then the cells were incubated for an additional 30 min at 37 °C. Finally, DCF-DA (5 μg/mL) was introduced to the cells, and 2′,7′-dichlorodihydrofluorescein fluorescence was detected at an excitation wavelength of 485 nm and an emission wavelength of 535 nm, using a Perkin-Elmer LS-5B spectrofluorometer (Waltham, MA, USA).

2.4 Origin and Maintenance of Parental Zebrafish

Ten adult zebrafish were obtained from a commercial dealer (Seoul aquarium, Seoul, Korea) and were kept in a 3 L acrylic tank at 28.5 °C with a 14:10 h light:dark cycle. The zebrafish were fed three times a day, 6 days/week, with tetramin flake food supplemented with live brine shrimps (Artemia salina; SEWHAPET Food Co.,

Seoul, Korea). Embryos were obtained from natural spawning that was induced in the morning by turning on the light. The collection of embryos was completed within 30 min.

2.5 Preparation of Inflammation-Induced Zebrafish Model by LPS Treatment and Application of Test Short Neck Clam Extract

Synchronized zebrafish embryos were collected and arrayed by pipette, 10–15 embryos/well, in 12-well plates containing 2 mL embryo medium for 7–9 h post-fertilization (hpf), and then incubated without or with the test samples for 1 h. To induce inflammation, 5 μg/mL LPS (final concentration) was added to the embryo medium for 15–17 hpf at 28.5 °C. Thereafter, the zebrafish embryos were transferred to fresh embryo medium.

2.6 Measurement of Inflammation-Induced Cell Death and Image Analysis

Cell death was detected in live embryos using acridine orange staining. Acridine orange stain cells with disturbed plasma membrane permeability, therefore, it preferentially stains necrotic or very late apoptotic cells. At 3 dpf, a zebrafish larva was transferred to one well of 96-well plates, treated with acridine orange solution (7 g/mL) and incubated for 30 min under the dark at 28.5 °C. After the incubation, the zebrafish larvae were rinsed by fresh embryo media and anaesthetized by 2-phenoxy ethanol (1/500 dilution sigma) before observation and photographed under the microscope CoolSNAP-Pro color digital camera (Olympus, Japan). Fluorescence intensity of individual zebrafish larva was quantified using the image J program.

2.7 Estimation of Inflammation-Induced Intracellular Reactive Oxygen Species (ROS) Generation and Image Analysis

The generation of ROS in inflammatory zebrafish model was analyzed using an oxidation-sensitive fluorescent probe dye, 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA), which is deacetylated intracellular by nonspecific esterase and is further oxidized to the highly fluorescent compound, dichlorofluorescein (DCF) in the presence of cellular peroxides (Rosenkranz et al. 1992). Following LPS treatment, the zebrafish larvae and embryos were transferred into 96-well plates, treated with DCF-DA solution (20 μg/mL), and incubated for 1 h in the dark at 28.5 °C.

After incubation, the zebrafish larvae and embryos were rinsed with fresh zebrafish embryo medium and anesthetized in tricaine methanesulfonate solution before observation. Individual zebrafish larvae and embryo fluorescence intensity was quantified using a spectrofluorometer (Perkin-Elmer LS-5B, Norwalk, CT, USA). The images of the stained larvae and embryos were observed using a fluorescent microscope, which was equipped with a Moticam color digital camera (Motix, Xiamen, China).

2.8 Estimation of Inflammation-Induced Intracellular Nitric Oxide (NO) Generation and Image Analysis

Generation of NO in inflammatory zebrafish model was analyzed using a fluorescent probe dye, diaminofluorophore 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAFFM DA). The transformation of DAFFM DA by NO in the presence of dioxygen generates highly fluorescent triazole derivatives (Itoh et al. 2000). Following LPS treatment, the zebrafish larvae and embryos were transferred into 96-well plates, treated with DAF-FM DA solution (5 μM), and incubated for 1 h in the dark at 28.5 °C. After incubation, the zebrafish larvae and embryos were rinsed in the fresh zebrafish embryo medium and anesthetized in tricaine methanesulfonate solution before observation. Individual zebrafish larvae and embryos fluorescence intensity was quantified using a spectrofluorometer (Perkin-Elmer LS-5B, Norwalk, CT, USA). The images of the stained larvae and embryos were observed using a fluorescent microscope, which was equipped with a Moticam color digital camera (Motix, Xiamen, China).

2.9 Statistical Analysis

All data are presented as the mean ± SEM. The data were evaluated by a one-way analysis of variance. Differences between the mean values were assessed using Tukey-Kramer multiple comparison test. Statistical significance was considered for values of $P < 0.05$.

3 Results

*3.1 Chemical Composition of Short Neck Clam (*Tapes philippinarum*)*

The chemical compositions of short neck clam are shown in Table 1. The contents of dry matter, crude protein, crude lipids and ash were 96.43 %, 58.25 %, 5.41 % and 6.50 %, respectively.

Table 1 Chemical composition of short neck clam (*Tapes philippinarum*)

Chemical composition (%)	
Dry matter	96.43 ± 0.43
Crude protein	58.25 ± 0.57
Crude lipids	5.41 ± 0.11
Ash	6.50 ± 0.13

Results are mean values of three replicates ± SD. Expressed as % (g per 100 g) on a dry weight basis

*3.2 Amino Acid Composition of Short Neck Clam (*Tapes philippinarum*)*

Table 2 shows the amino acid compositions of short neck clam. In the present study, total amino acid concentration was 7.81-fold higher in the short neck clam water extract compared to the lysis buffer extract. Glycine and taurine were the most abundant free amino acids of short neck clam water extract. Especially, taurine concentration of short neck clam water extract was 366.83 nmol/mg, which was 8.24-fold higher compared to that of lysis buffer extract. Therefore, we used short neck clam water extract for anti-inflammatory effect on the LPS-stimulated zebrafish model in the following experiments.

3.3 Protective Effect of Short Neck Clam Extract Against LPS-Induced Cell Death in the In Vitro *and* In Vivo *Zebrafish Model*

Cell viabilities associated with LPS treatment or with co-treatment with LPS and short neck clam water extract are shown in Fig. 1a. The cell viability of non-treated cells was assigned as 100 %, and the cell viability was reduced in the LPS-treated cells. On the other hand, the short neck clam water extract treatment tended to decrease cell viability as compared with those in the control group. This result indicates that short neck clam water extract has no protective effect against oxidative stress in cells *in vitro*. Figure 1b shows that the cell death induced by LPS treatment. However, cell death was reduced by adding short neck clam water extract to LPS-treated zebrafish.

3.4 Inhibitory Effect of Short Neck Clam Extract on LPS-Stimulated ROS Generation in Inflammatory Zebrafish Model

Figure 2 shows the inhibitory effect of short neck clam water extract on LPS-induced ROS generation in zebrafish model. Figure 2a is a typical fluorescence micrograph of the zebrafish. The negative control, which contained no short neck

Table 2 Amino acid composition of short neck clam (*Tapes philippinarum*)

Amino acid composition (nmol/mg)	Water extract	Lysis buffer extract
Cysteine	1.47	0.00
Aspartic acid	31.18	4.69
Glutamic acid	86.49	11.48
Asparagine	2.71	0.00
Serine	20.18	2.88
Glutamine	15.61	2.15
Glycine	464.83	58.53
Histidine	14.53	1.24
Arginine	67.78	9.90
Taurine	366.83	44.52
Threonine	22.23	3.14
Alanine	189.51	26.66
Proline	21.79	3.14
Tyrosine	8.93	1.52
Valine	21.25	2.76
Methionine	9.72	0.62
Isoleucine	12.86	1.45
Leucine	17.85	2.25
Phenylalanine	11.15	1.32
Tryptophan	4.23	0.44
Lysine	21.22	2.21
Total	1412.37	180.92

clam extract or LPS treatment, generated a clear image, whereas the positive control, the LPS treatment group, generated a fluorescence image, which suggests that ROS took place during LPS treatment of the zebrafish. However, in zebrafish that were treated with short neck clam extract prior to LPS treatment, a dramatic reduction in the amount of ROS was observed. Short neck clam extract therefore markedly attenuated the increase in ROS levels induced by LPS treatment in the zebrafish model. In the present study, we also confirmed that the pretreatment with short neck clam water extract together with LPS significantly inhibited ROS generation in a dose-dependent manner, indicating protection against ROS (Fig. 2b). Our results demonstrate that treating zebrafish with LPS-treatment significantly increased ROS levels. However, short neck clam extract possessing taurine inhibited LPS-induced ROS generation. These results show that short neck clam extract alleviated inflammation by inhibiting ROS generation induced by LPS treatment.

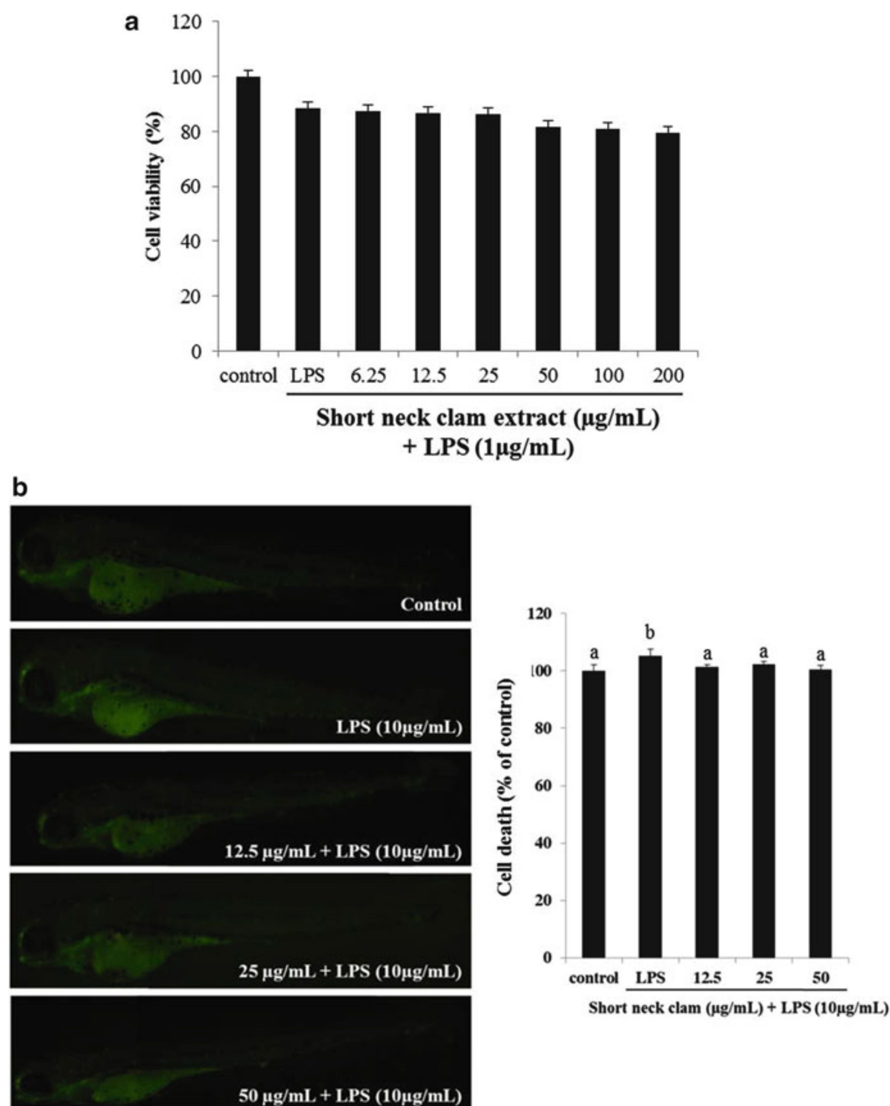

Fig. 1 Protective effects of short neck clam extract against LPS-induced cell death in Vero cells (**a**). Measurement of LPS toxicity and LPS-co treated with short neck clam extract on cell death (**b**). Experiments were performed in triplicate and data are means ± SEM. Values not sharing a common letter are significantly different at $P < 0.05$ by Turkey-Kramer multiple comparison test

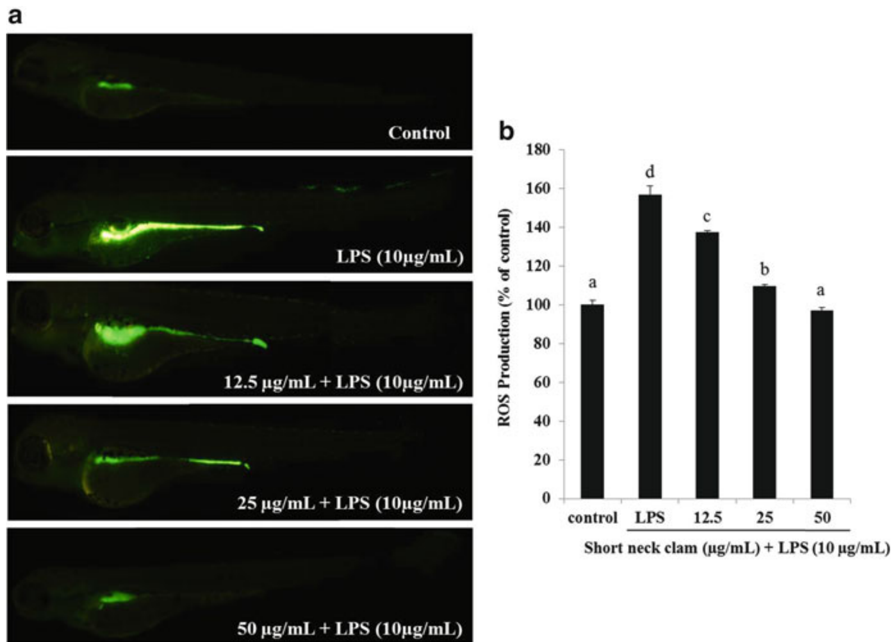

Fig. 2 Inhibitory effect of short neck clam extract on LPS-induced ROS generation in zebrafish larvae. ROS generation was determined by the extent of DCF-DA staining. (**a**) Fluorescence micrographs of LPS-induced ROS generation. (**b**) A fluorescence spectrophotometer was used for the quantitative analysis of ROS generation. Experiments were performed in triplicate and the data are expressed as means ± SEM. Values not sharing a common letter are significantly different at $P < 0.05$ by Turkey-Kramer multiple comparison test

3.5 Inhibitory Effect of Short Neck Clam Extract on LPS-Stimulated NO Generation in Inflammatory Zebrafish Model

Figure 3 shows the inhibitory effect of short neck clam water extract on LPS-induced NO generation in zebrafish. The level of NO in zebrafish was significantly elevated by the LPS treatment as compared with the non-LPS treated zebrafish (negative control). However, the NO level in the short neck clam extract-treated zebrafish was dose-dependently and significantly reduced in the range of 6.25–100 μg/mL. These results indicated that short neck clam extract significantly attenuate the increase in NO levels induced by LPS treatment in the zebrafish model.

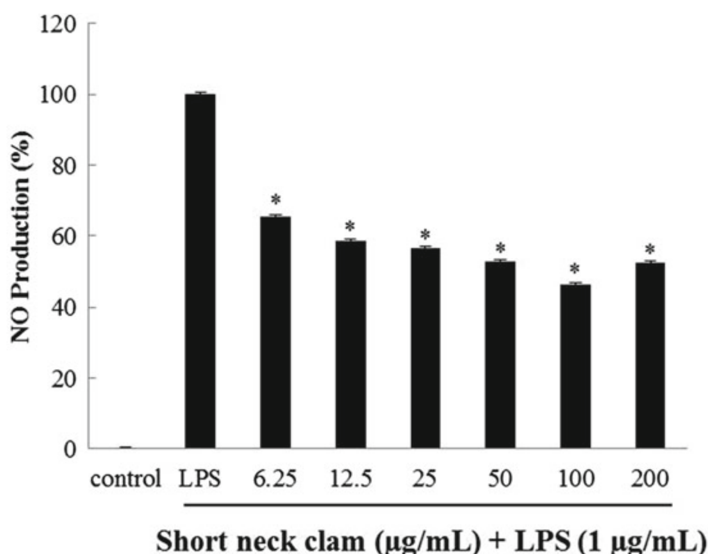

Fig. 3 Inhibitory effect of short neck clam extract on LPS-induced NO generation in zebrafish larvae. The NO generation level was measured after staining with DAF-FM-DA. A fluorescence spectrophotometer was used for quantitative analysis of NO generation. Experiments were performed in triplicate and the data are expressed as means ± SEM. *$P < 0.05$ shows a significant difference from the only LPS-treated zebrafish by the Turkey-Kramer multiple comparison test

4 Discussion

Inflammation is a complex physiological response of tissues to harmful stimuli such as pathogens, damaged cells or cancer cells and irritants. Especially, chronic and uncontrolled inflammation is detrimental to tissue, which may cause chronic inflammation-derived diseases, such as cardiovascular diseases and cancers (Karin et al. 2006; Marcinkiewicz and Kontny 2014). Recent studies have reported that the zebrafish model can be used to rapidly and simply assess the anti-inflammatory activity on LPS-stimulated inflammation *in vivo* (Park and Cho 2011). In the present study, we first investigated the *in vivo* anti-inflammatory effect of the water extract from short neck clam using the zebrafish model.

In our present study, we have confirmed that taurine and glycine are the most abundant total free amino acids of the water extract of short neck clam. Especially, it has been reported that taurine plays and an important role in the immune system as an antioxidant to protect cells, including leukocytes, from oxidative stress (Wang et al. 2009). In the present study, cell death was significantly reduced by adding short neck clam water extract possessing high amounts of taurine to LPS-treated

zebrafish, while short neck clam water extract has no protective effects on LPS-induced cell death in Vero cells. In some previous studies, taurine chloramine (TauCl) inhibited NO, PGE2, TNF-a, and IL-6 production from stimulated macrophages in culture and the capacity for proinflammatory cytokine production, producing an anti-inflammatory effect. However, taurine has no anti-inflammatory effect in vitro (Grimble 1996; Huxtable 1996).

In the inflammatory process, oxidative stress is mediated by reactive oxygen species (ROS) generated primary by activated leukocytes. ROS plays a beneficial role in host defense against pathogens, but they are also responsible for tissue injury (Smith 1994). Previous studies have indicated that high ROS levels induce oxidative stress which can result in several biochemical and physiological lesions. Such cellular damage frequently impairs metabolic function and results in cell death and inflammation of tissues (Finkel and Holbrook 2000). Other previous studies have demonstrated that taurine has the capability of scavenging a particular compound, hypochlorous acid, an oxidant that activates the tyrosine kinase signaling cascade that leads to the formation of inflammatory mediators (Marcinkiewicz et al. 2000; Schaffer et al. 2009). Our results demonstrate that LPS treated zebrafish significantly increase ROS levels. However, short neck clam water extract markedly inhibits LPS-induced ROS generation. These results show that short neck clam water extract containing large amounts of taurine alleviate inflammation by inhibiting ROS generation induced LPS treatment. Similarly, it was reported that taurine inhibits inflammation and oxidative stress while increasing NO generation (Katama et al. 1996; Sener et al. 2005). Another previous study reported that Pacific oyster extract contains potent antioxidant activity in the cell-based antioxidant assay (Watanabe et al. 2012).

In general, NO is a reactive oxygen species which is increased by the inducible NOS (iNOS), subsequently, brings about cytotoxicity, and tissue damage (Kim et al. 1999). Therefore, NO inhibitors are essential for the prevention of inflammatory diseases. In the present study, we confirmed that the level of NO in zebrafish was significantly elevated by the LPS treatment as compared with non-LPS treated zebrafish. However, the water extract from short neck clam dose-dependently and significantly reduced the elevated NO level induced by LPS in inflammatory zebrafish model. Similar with our results, Huang et al. (2013) reported that freshwater clam extract supplementation also reduced inflammatory stress. Another previous study also reported that taurine treatment significantly attenuated cellular and biochemical alterations induced by LPS in hamsters (Bhavsar et al. 2009). More recently, it has been reported that taurine supplementation significantly reduces inflammatory markers, such as hs-C-reactive protein and lipid peroxidation markers, such as thiobarbituric acid reactive substances (TBARS) in human study, and these results are related to the amino acids composition of cod fish, especially taurine (Rosa et al. 2014). These findings indicate that short neck clam water extract possessing taurine might confer important protection against inflammation induced by physical and chemical damage in the zebrafish model.

5 Conclusion

Our present study shows that the water extract of short neck clam exhibits potent anti-inflammatory properties against LPS treatment-induced inflammation. The short neck clam water extract also has a protective effect against the toxicity induced by LPS exposure in zebrafish embryos. The short neck clam exhibit profound anti-inflammatory effect both *in vitro* as well as *in vivo*, suggesting that the short neck clam extract containing taurine might be a potent anti-inflammatory agent.

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In Vitro and *In Vivo* Antioxidant and Anti-inflammatory Activities of Abalone (*Haliotis discus*) Water Extract

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Abbreviations

AAPH	2,2′-azobis dihydrochloride
DPPH	1,1-diphenyl-2-picrylhydrazyl
ESR	electron spin resonance
LPS	lipopolysaccharide
NO	nitric oxide
ROS	reactive oxygen species

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1 Introduction

Taurine is a neutral β-amino acid, which is not utilized in protein synthesis but found mostly in free form in marine mollusks as well as the human body (Redmond et al. 1998). Taurine has been reported to possess various biological activities, such as osmoregulation, neurotransmission, and membrane stabilization (Hansen 2001). Moreover, taurine is a protective agent against oxidative stress-induced pathologies, such as atherosclerosis, diabetes mellitus and apoptosis (Hansen 2001; Balkan et al. 2002; Cetiner et al. 2005). Taurine is found at particularly high concentration in tissues exposed to elevated levels of oxidants, suggesting its role in the attenuation of oxidative stress (Oliveira et al. 2010).

Oxidative stress caused by reactive oxygen species (ROS) is well known to contribute to the progression several of oxidant-related diseases, including inflammatory diseases, cardiovascular diseases, neurological disorders and cancers (Yang et al. 2001; Camuesco et al. 2004; Stocker and Keaney 2004; Zhu et al. 2004). At present, some synthetic antioxidants such as butylated hydroxytolluene (BHT) and butylated hydroxyanisole (BHA) are commonly used to neutralize free radicals in biological systems, as well as in the food industry. However, the use of these artificial antioxidants is under regulation, due to risks related to health problems (Peng et al. 2009). Therefore, recent studies have focused on crude extracts or isolated antioxidants from marine organisms, such as algae and marine sponges (Takamatsu et al. 2003; Utkina et al. 2004). Some researchers demonstrated that enzymatic hydrolysates and peptides isolated from marine organisms exert antioxidant effects without untoward side effects (Chen et al. 2012; Wang et al. 2013). However, the anti-inflammatory and antioxidant potentials of abalone water extract possessing high amounts of taurine have not been reported.

The vertebrate zebrafish (*Danio rerio*) is a small tropical freshwater fish, which has emerged as an useful vertebrate model organism because of small size, large clutches, transparency, low cost maintenance, morphological and physiological similarity to mammals (Eisen 1996). Taken together, the objective of the present study was to evaluate the *in vitro* and *in vivo* anti-inflammatory, as well as antioxidant effect, of the water extract from abalone in zebrafish model.

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2 Methods

2.1 Reagents

5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO), 2,2-azobis (2-amidinopropane) hydrochloride (AAPH), 1,1-diphenyl-2-picrylhydrazyl (DPPH) and *alpha*-(4-pyridyl-1-oxide)-*N-tert*-butylnitrone (4-POBN) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of the highest grade available commercially.

2.2 Measurement of Free Radical Scavenging Activity

2.2.1 DPPH Radical Scavenging Activity

DPPH radical scavenging activity was measured using the method described by Nanjo et al. (1996). Briefly, 60 μL of abalone extract at each concentration was added to 60 μL of DPPH (60 μM) in a methanol solution. After mixing vigorously for 10 s, the solution was transferred into a 100 μL Teflon capillary tube, and the scavenging activity of each sample for the DPPH radical was measured using an ESR spectrometer. A spin adduct was measured on an ESR spectrometer exactly 2 min later. The experimental conditions were as follows: central field, 3,475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 5 mW; gain, 6.3×10^5 and temperature, 298 K.

2.2.2 Superoxide Radical Scavenging Activity

Superoxide radicals were generated by UV irradiation of a riboflavin/ethylenediaminetetraacetic acid solution. The reaction mixtures containing 0.1 mL of 0.8 mM riboflavin, 0.1 mL of 1.6 mM EDTA, 0.1 mL of 800 mM DMPO and 0.1 mL sample were irradiated for 1 min under a UV lamp at 365 nm. The mixtures were transferred to a 100 μL quartz capillary tube and placed in an ESR spectrometer for measurement. Experimental conditions were as follows: central field, 3,475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 10 mW; gain, 6.3×10^5 and temperature, 298 K.

2.2.3 Alkyl Radical Scavenging Activity

Alkyl radicals were generated by AAPH. The phosphate-buffered saline (pH 7.4) reaction mixtures containing 10 mM AAPH, 10 mM 4-POBN, and the indicated concentrations of tested samples were incubated at 37 °C in a water bath for 30 min and then transferred to a 100 μL quartz capillary tube. The spin adduct was recorded

on an ESR spectrometer. Measurement conditions were as follows: central field, 3,475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 1 mW; gain, 6.3×10^5; and temperature, 298 K.

2.3 Chemical Analysis and Amino Acid Composition

The abalone sample was analyzed for crude protein, crude fat (ether extract) and total ash content using the methods of the Association of Official Analytical Chemists (AOAC, 1990). For amino acid analysis, abalone samples (80 mg) were combined with 10 mL of 6 N HCl. After N_2 gas was used to purge the samples in the test tube, the samples were hydrolysed in a dry oven at 110 °C for 24 h. The hydrolysed samples were then evaporated and a sodium-distilled buffer (pH 2.2) was added. Samples were then filtered through a syringe filter (0.45 μm) and analyzed using an amino acid autoanalyzer (Pharmacia Biotech Biochrom 20, Ninhydrin Method). Amino acids were determined by absorbance at 440 and 570 nm.

2.4 In Vivo *Zebrafish Model*

2.4.1 Origin and Maintenance of Parental Zebrafish

Adult zebrafish were obtained from a commercial dealer (Seoulaquarium, Seoul, Korea) and 15 fish were kept in 3.5 L acrylic tank with the following conditions; 28.5 ± 1 °C, and were fed twice a day (Tetra GmgH D-49304 Melle Made in Germany) and subjected to a 14/10 h light/dark cycle. The day before, breeding one female and two males interbreed. In the morning (on set of light), embryos were obtained from the natural spawning collection of embryos were completed within 30 min in petri dishes containing media.

2.4.2 Waterborne Exposure of Embryos to AAPH

The embryos (n = 15) were transferred to individual wells of 12-well plates containing 900 L embryo media from approximately 7–9 hpf, After the incubation for 1 h, 15 mM AAPH solution was added to embryo medium and the embryo were exposed to AAPH for up to 24 hpf. Then, embryos were rinsed using fresh embryo media.

2.4.3 Measurement of Oxidative Stress-Induced Intracellular ROS Generation and Image Analysis

Generation of ROS production of zebrafish was analyzed using an oxidation-sensitive fluorescent probe dye, DCFH-DA. At 3 dpf, a zebrafish larva was transferred to one well of a 96-well plate, treated with DCFH-DA solution (20 g/mL) and

incubated for 1 h in the dark at 28.5 ± 1 °C. After the incubation, the zebrafish larvae were rinsed by fresh embryo media and anaesthetized with 2-phenoxy ethanol (1/500 dilution sigma) before observation and photographed under the microscope CoolSNAP-Pro color digitalcamera (Olympus, Japan). A fluorescence intensity of individual larva was quantified using the image J program.

2.4.4 Measurement of Oxidative Stress-Induced Cell Death and Image Analysis

Cell death was detected in live embryos using acridine orange staining. Acridine orange stain cells with disturbed plasma membrane permeability, therefore, it preferentially stains necrotic or very late apoptotic cells. At 3 dpf, a zebrafish larva was transferred to one well of 96-well plates, treated with acridine orange solution (7 g/mL) and incubated for 30 min under the dark at 28.5 ± 1 °C. After the incubation, the zebrafish larvae were rinsed by fresh embryo media and anaesthetized with 2-phenoxy ethanol (1/500 dilution sigma) before observation and photographed under the microscope CoolSNAP-Pro color digital camera (Olympus, Japan). Fluorescence intensity of individual zebrafish larva was quantified using the image J program.

2.5 Cell Culture

The cells of an African green monkey kidney (Vero) were maintained at 37 °C in an incubator, under a humidified atmosphere containing 5 % CO_2. The cells were cultured in DMEM containing 10 % heat-inactivated FBS, streptomycin (100 μg/mL), penicillin (100 unit mL^{-1}), and sodium pyruvate (110 mg L^{-1}).

2.6 Intracellular LPS Scavenging Assay by DCF-DA

For the detection of intracellular LPS (1 μg/mL), the Vero cells were seeded in 96-well plates at a concentration of 1.0×10^5 cells mL^{-1}. After 16 h, the cells were treated with samples (10 μL) and incubated at 37 °C under a humidified atmosphere. After 30 min, LPS was added at a concentration of 1 μg/mL, and then the cells were incubated for an additional 30 min at 37 °C. Finally, DCF-DA (5 μg/mL) was introduced to the cells, and 2′,7′-dichlorodihydrofluorescein fluorescence was detected at an excitation wavelength of 485 nm and an emission wavelength of 535 nm, using a Perkin-Elmer LS-5B spectrofluorometer (Waltham, MA, USA).

2.7 Preparation of Inflammation-Induced Zebrafish Model by LPS Treatment and Application of Test Abalone Extract

Synchronized zebrafish embryos were collected and arrayed by pipette, 10–15 embryos/well, in 12-well plates containing 2 mL embryo medium for 7–9 h post-fertilization (hpf), and then incubated without or with the test samples for 1 h. To induce inflammation, 5 μg/mL LPS (final concentration) was added to the embryo medium for 15–17 hpf at 28.5 °C. Thereafter, the zebrafish embryos were transferred in the fresh embryo medium.

2.8 Measurement of Inflammation-Induced Cell Death and Image Analysis

Cell death was detected in live embryos using acridine orange staining. Acridine orange stains cells with disturbed plasma membrane permeability, therefore, it preferentially stains necrotic or very late apoptotic cells. At 3 dpf, a zebrafish larva was transferred to one well of 96-well plates, treated with acridine orange solution (7 g/mL) and incubated for 30 min under the dark at 28.5 °C. After the incubation, the zebrafish larvae were rinsed by fresh embryo media and anaesthetized with 2-phenoxy ethanol (1/500 dilution sigma) before observation and photographed under the microscope CoolSNAP-Pro color digital camera (Olympus, Japan). A fluorescence intensity of individual zebrafish larva was quantified using the image J program.

2.9 Estimation of Inflammation-Induced Intracellular Reactive Oxygen Species (ROS) Generation

The generation of ROS in inflammatory zebrafish model was analyzed using an oxidation-sensitive fluorescent probe dye, 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA). The DCF-DA is deacetylated intracellular by nonspecific esterase and is further oxidized to the highly fluorescent compound, dichlorofluorescein (DCF) in the presence of cellular peroxides (Rosenkranz et al. 1992). Following LPS treatment, the zebrafish larvae and embryos were transferred into 96-well plates, treated with a DCF-DA solution (20 μg/mL), and incubated for 1 h in the dark at 28.5 °C. After incubation, the zebrafish larvae and embryos were rinsed in the fresh zebrafish embryo medium and anesthetized in tricaine methanesulfonate solution before observation. Individual zebrafish larvae and embryo fluorescence intensity was quantified using a spectrofluorometer (Perkin-Elmer LS-5B, Norwalk, CT, USA). The images of the stained larvae and embryos were observed using a fluorescent microscope, which was equipped with a Moticam color digital camera (Motix, Xiamen, China).

2.10 Estimation of Inflammation-Induced Intracellular Nitric Oxide (NO) Generation

Generation of NO in the inflammatory zebrafish model was analyzed using a fluorescent probe dye, diaminofluorophore 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAFFM DA). Transformation of DAFFM DA by NO in the presence of dioxygen generates highly fluorescent triazole derivatives (Itoh et al. 2000). Following LPS treatment, the zebrafish larvae and embryos were transferred into 96-well plates, treated with DAFFM DA solution (5 μM), and incubated for 1 h in the dark at 28.5 °C. After incubation, the zebrafish larvae and embryos were rinsed in the fresh zebrafish embryo medium and anesthetized with tricaine methanesulfonate before observation. Individual zebrafish larvae and embryos fluorescence intensity was quantified using a spectrofluorometer (Perkin-Elmer LS-5B, Norwalk, CT, USA). The images of the stained larvae and embryos were observed using a fluorescent microscope, which was equipped with a Moticam color digital camera (Motix, Xiamen, China).

2.11 Statistical Analysis

All data are presented as the means ± SEM. The data were evaluated by a one-way analysis of variance. Differences between the mean values were assessed using the Tukey-Kramer multiple comparison test. Statistical significance was considered for values of $P < 0.05$.

3 Results

3.1 Chemical Composition of Abalone (Haliotis discus)

Table 1 shows the chemical composition of abalone. In the present study, the content of dry matter was 98.11 %. Also, the content of crude protein, crude lipids and ash was 71.10 %, 2.58 % and 8.97 %, respectively.

3.2 Amino Acid Composition of Abalone (Haliotis discus)

The amino acid composition of abalone is shown in Table 2. In the present study, total amino acid concentration of abalone water extract was 9.71-fold higher compared to that of the lysis buffer extract. Taurine was the most abundant free amino acid in both water and lysis buffer extract from abalone. The taurine concentration

Table 1 Chemical composition of abalone (*Haliotis discus*)

Chemical composition (%)	
Dry matter	98.11 ± 0.65
Crude protein	71.10 ± 0.39
Crude lipids	2.58 ± 0.12
Ash	8.97 ± 0.11

Results are mean values of three replicates ± SD. Expressed as % (g per 100 g) on a dry weight basis

Table 2 Amino acid composition of abalone (*Haliotis discus*)

Amino acid composition (nmol/mg)	Water extract	Lysis buffer extract
Aspartic acid	12.33	2.53
Glutamic acid	52.22	5.84
Asparagine	0.00	0.00
Serine	34.99	3.78
Glutamine	12.75	0.96
Glycine	100.18	10.69
Histidine	6.55	0.42
Arginine	109.43	12.56
Taurine	593.80	57.58
Threonine	25.22	2.40
Alanine	55.15	6.15
Proline	20.50	2.36
Tyrosine	10.42	1.43
Valine	18.29	2.20
Methionine	2.50	0.31
Cysteine	0.00	0.00
Isoleucine	10.40	1.08
Leucine	12.27	1.51
Phenylalanine	8.14	0.86
Tryptophan	9.57	0.63
Lysine	20.89	1.54
Total	1115.60	114.84

of water extract was 593.80 nmol/mg, which is 10.3-fold higher compared to that of the lysis buffer extract. Therefore, the anti-inflammatory and antioxidant effects were examined in the following experiments.

3.3 Radical Scavenging Activities of Abalone Extract

In the present study, DPPH, superoxide and alkyl radical scavenging activities of abalone extract were measured using an ESR spectrometer. As shown in Fig. 1, the concentration of water extract from abalone that inhibited 50 % of free radical

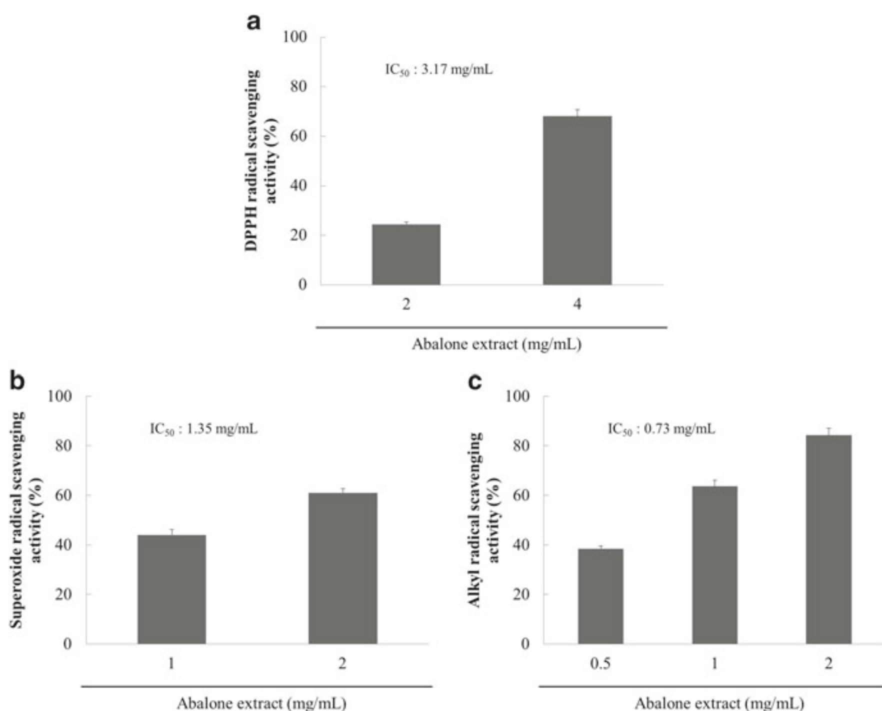

Fig. 1 Measurement of free radical scavenging activities of abalone extract by ESR. (**a**) DPPH scavenging activity, (**b**) superoxide radical scavenging activity and (**c**) alkyl radical scavenging activity. Values are means ± SD of triplicate experiments

(DPPH radical) generation (IC_{50}) was 3.17 (Fig. 1a). On the other hand, superoxide and alkyl radical scavenging activities were noted at water abstract concentrations of 1.35 mg/mL and 0.73 mg/mL, respectively (Fig. 1b, c).

3.4 Measurement of Abalone Extract Against AAPH-Induced Oxidative Stress In Vivo *Zebra Fish Model*

The cell death induced by AAPH treatment in zebrafish is shown in Fig. 2. AAPH-induced cell death in zebrafish was measured to 134 % compared to the control group. However, cell death was markedly reduced by adding abalone extract to AAPH-treated zebrafish in the range of 12.5–50 μg/mL ($P<0.05$).

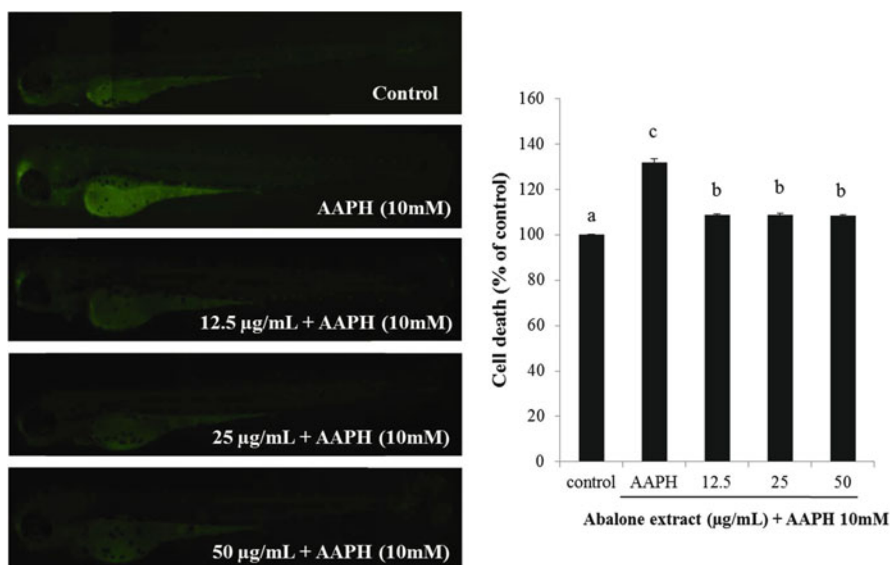

Fig. 2 Measurement of AAPH toxicity and AAPH-co treated with abalone extract on cell death. Experiments were performed in triplicate and data are means ± SEM. Values not sharing a common letter are significantly different at $P < 0.05$ by the Turkey-Kramer multiple comparison test

3.5 *Protective Effect of Abalone Extract Against LPS-Induced Cell Death in the* In Vitro *and* In Vivo *Zebrafish Model*

We also measured cell viability after treatment with LPS or co-treatment with abalone water extract in Vero cells and the results are shown in Fig. 3a. Cell viability of the non-treated cells was assigned a value of 100 %, and the cell viability was reduced in the LPS-treated cells. The abalone water extract treatment tended to decrease cell viability as compared with those in the control group. This result indicates that abalone water extract has no protective effect against ROS oxidation stress in Vero cells. Figure 3b shows that cell death induced by LPS treatment in zebrafish. LPS-induced cell death in zebrafish was increased compared to the control group. However, cell death was dose-dependently reduced by adding abalone water extract to LPS-treated zebrafish.

3.6 *Inhibitory Effect of Abalone Extract on LPS-Stimulated ROS Generation in Inflammatory Zebrafish Model*

Figure 4 shows the inhibitory effect of abalone water extract on LPS-induced ROS generation in zebrafish model. Figure 4a is a typical fluorescence micrograph of the zebrafish. The negative control, which contained no abalone extract or LPS

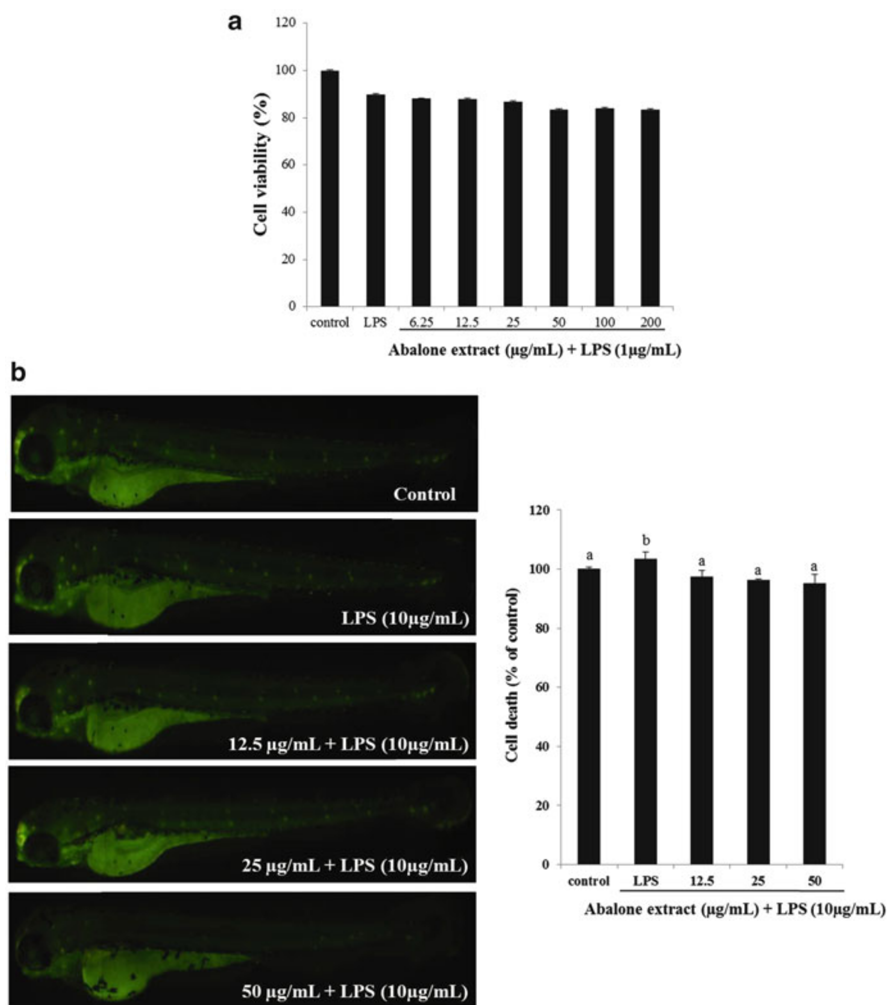

Fig. 3 Protective effects of abalone against LPS-induced cell death in Vero cells (**a**). Measurement of LPS toxicity and beneficial effect of abalone extract on cell death (**b**). Experiments were performed in triplicate and data are means ± SEM. Values not sharing a common letter are significantly different at $P < 0.05$ by the Turkey-Kramer multiple comparison test

treatment, generated a clear image, whereas the positive control, which was only LPS treatment, generated a fluorescence image, which suggests that ROS took place during LPS treatment of the zebrafish. However, in zebrafish that were treated with abalone water extract prior to LPS treatment, a dramatic reduction in the amount of ROS was observed. In the present study, we confirmed that pretreatment with abalone water extract together with LPS significantly inhibited ROS generation in a dose-dependent manner, indicating protection against ROS (Fig. 4b). Our results

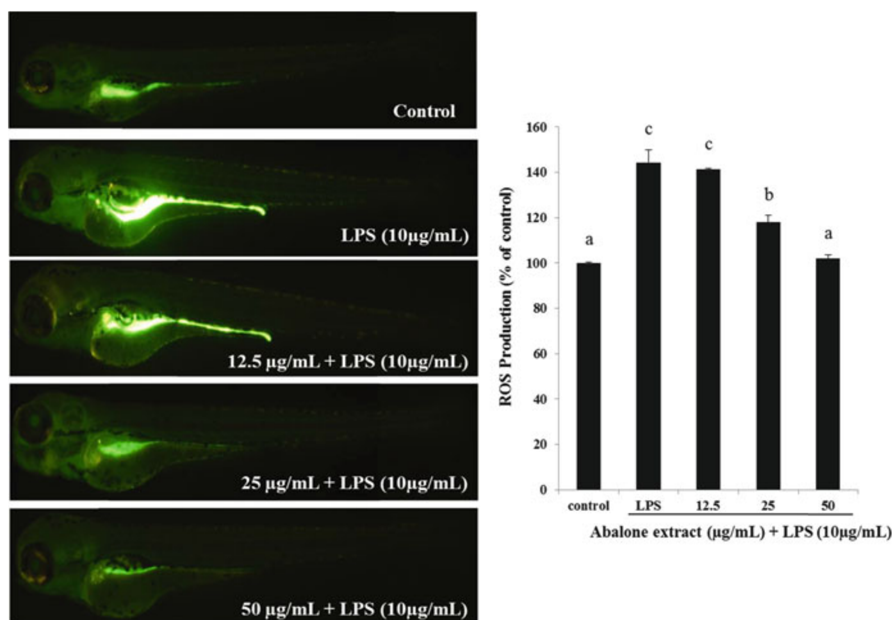

Fig. 4 Inhibitory effect of abalone extract on LPS-induced ROS generation in zebrafish larvae. The ROS generation level was measured after staining with DCF-DA. (**a**) Fluorescence micrographs of LPS-induced ROS generation. (**b**) A fluorescence spectrophotometer was used for the quantitative analysis of ROS generation. Experiments were performed in triplicate and the data are expressed as means ± SEM. Values not sharing a common letter are significantly different at $P<0.05$ by the Turkey-Kramer multiple comparison test

demonstrate that treating zebrafish with LPS-treatment significantly increased ROS level. However, abalone extract possessing amounts of taurine inhibited LPS-induced ROS generation.

3.7 Inhibitory Effect of Abalone Extract on LPS-Stimulated NO Generation in Inflammatory Zebrafish Model

Figure 5 shows the inhibitory effect of abalone water extract on LPS-induced NO generation in zebrafish. The level of NO in zebrafish was significantly elevated by LPS treatment compared with the non-LPS treated zebrafish (negative control). However, the NO level in the abalone extract-treated zebrafish was dose-dependently and significantly reduced in the range of 6.25–100 μg/mL. These results indicated that abalone extract significantly attenuates the increase in NO levels induced by LPS treatment in the zebrafish model.

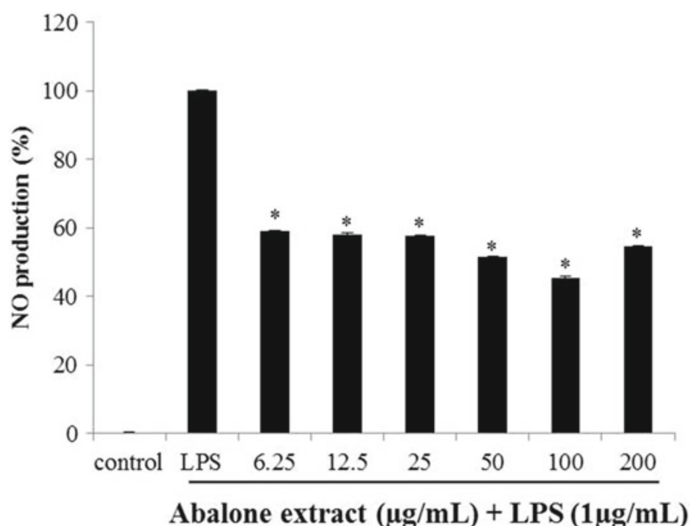

Fig. 5 Inhibitory effect of abalone extract on LPS-induced NO generation in zebrafish larvae. The generation of NO was measured after staining with DAF-FM-DA. A fluorescence spectrophotometer was used for the quantitative analysis of NO generation. Experiments were performed in triplicate and the data are expressed as means ± SEM. $^{*}P < 0.05$ shows significant difference from the only LPS-treated zebrafish by the Turkey-Kramer multiple comparison test

4 Discussion

Oxidative stress increases with accumulation of free radical species including hydroxyl and alkyl radicals and decreases with the defense provided by antioxidants (Lu et al. 2010). Antioxidant responses of living organisms are the important defense mechanisms to resist the exposure of toxicants. Peroxidation by reactive oxygen species (ROS) can lead to the formation of lipid peroxides, such as malondialdehyde (MDA); increases in peroxidation suggests a cytotoxic event and/or inhibition of the antioxidant enzymes (Emmanouil et al. 2008). On the other hand, it is well known that taurine, which is abundant in several marine organisms such as molluscs, crabs and fish, protects cells against oxidative injury (Schaffer et al. 2009). Although a number of studies concerning the therapeutic effect of taurine have been reported, there is still little known about the potential effect of abalone extract containing amounts of taurine on antioxidant and anti-inflammatory activities *in vivo* zebrafish model. Recently, zebrafish model has been commonly used to model human diseases and processes such as oxidative stress, liver damage and inflammation (Kang et al. 2013). In our present study, taurine is the most abundant free amino acid in both water and lysis buffer extract from abalone. The abalone water extract exhibits free radical scavenging activity against DPPH, superoxide and alkyl radical *in vitro*. Similarly, Wang et al. (2013) indicated that blue mussel

hydrolysate shows concentration-dependent anti-DPPH, hydroxyl ad superoxide radical activities.

In general, AAPH-induced oxidative stress leads to cell damage and eventually cell death (Kang et al. 2013). In the present study, however, cell death was significantly reduced by adding abalone water extract possessing high amount of taurine to AAPH-treated zebrafish, although, abalone water extract has no protective effects against LPS-induced cell death in Vero cells. Nonetheless a number of studies have reported the effects of metabolic products of sulfur amino acids such as taurine in *in vitro* studies involving various functions of human immune cells. Some *in vitro* studies, however, have shown that increases in taurine concentration from physiological to super-physiological concentrations have no effect on proinflammatory cytokine production by peripheral blood mononuclear cells (Grimble 1996; Huxtable 1996). However, taurine chloramine (TauCl) inhibits nuclear factor kB activation and the capacity for proinflammatory cytokine production, producing an antiinflammatory effect (Huxtable 1996).

In the present study, we also evaluated antioxidant and anti-inflammatory effect of abalone water extract using lipopolysaccharide (LPS)-stimulated zebrafish model. Generally, it is known that LPS along with oxidase-expressing epithelial and endothelial cells are considered to be major proinflammatory factors owing to their ability to activate neutrophils to generate ROS (Bhavsar et al. 2009). In the present study, we confirmed that LPS-treatment markedly increased ROS content. However, abalone water extract dose-dependently and significantly inhibited LPS-induced ROS generation. These results show that abalone extract containing high amounts of taurine alleviated inflammation by inhibiting ROS generation induced by LPS treatment. Some previous studies have indicated that taurine is able to sequester ROS such as peroxyl and superoxide radicals and reactive nitrogen species such as peroxynitrite and protect against lipid peroxidation (Xiao et al. 2008; Oliveira et al. 2010). Recently, it was reported that taurine supplement restores superoxide dismutase (SOD) and catalase activities and markedly reduce lipid peroxidation after alcohol exposure in zebrafish (Rosemberg et al. 2010). Kalaz et al. (2014) reported that taurine treatment has a protective effect against galactose-induced oxidative stress and tissue injury in rat liver.

On the other hand, it has been reported that the anti-inflammatory action seems to be related to the antioxidant activity of taurine (Marcinkiewicz et al. 1995). Especially, NO is an important inflammatory mediator of ROS generation and thus the alteration of nitric oxide level was often estimated to determine macrophage activity (Hibbs et al. 1988). Several previous studies have shown that NO production is increased by activation of inducible nitric oxide synthase (iNOS) mRNA expression in spleen and other principal organs after LPS treatment (Molina et al. 1998; Lin et al. 2006). Therefore, we evaluated in this study the inhibitory effect of abalone water extract on LPS-induced NO production in zebrafish by using a fluorescent probe dye, DAF-FM DA. In the present study, we confirmed that NO content in abalone water extract-treated zebrafish was reduced significantly. Similarly, a previous study reported that taurine showed protective effect against LPS-induced oxidative stress, and significantly reduced NO-mediated damage (Bircan et al. 2011).

Overall, these results suggest that abalone water extract acts as a potent inhibitor of ROS and NO in LPS-stimulated inflammatory zebrafish model. In addition, abalone water extract has a protective effect against toxicity induced by AAPH, as well as LPS exposure in zebrafish embryos. This outcome could explain the potential antioxidant and anti-inflammatory activities of abalone water extract possessing taurine, which might have a beneficial effect during the treatment of inflammatory diseases related to oxidative stress.

5 Conclusion

In conclusion, the results obtained in the present study show that abalone water extract, which contain substantial amounts of taurine could effectively inhibit cell death induced by AAPH. In addition, we investigated the protective effects of the abalone water extract against LPS-induced oxidative stress in zebrafish embryos. Our results demonstrate that LPS induces toxicity in zebrafish embryos and abalone water extract can protect zebrafish embryos against LPS, by inhibiting intracellular ROS formation, cell death and NO formation. Therefore, abalone water extract has anti-inflammatory, as well as antioxidant activity in the zebrafish model.

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Effect of *N*-(D-Ribopyranosyl)taurine Sodium Salt Supplementation on the Hepatic Antioxidant System in Rat Models of Diet-Induced Obesity and Taurine Deficiency

So Young Kim, Jeong Soon You, Yun Ju Lee, Kyung Ja Chang, Hye Jeong Cho, and Sung Hoon Kim

Abbreviations

T-Rib	*N*-(D-Ribopyranosyl)taurine sodium salt
N	Normal diet
HF	High-fat diet
HFT	High-fat diet + taurine
HFA	High-fat diet + β-alanine
HFR2	High-fat diet + β-alanine + T-Rib (2 mmol/kg/day)
HFR4	High-fat diet + β-alanine + T-Rib (4 mmol/kg/day)
HFR6	High-fat diet + β-alanine + T-Rib (6 mmol/kg/day)
GOT	Glutamate oxaloacetate transaminase
GPT	Glutamate pyruvic transaminase
TBARS	Thiobarbituric acid reactive substance
GSH	Glutathione
GPx	Glutathione peroxidase
SE	Standard error
H&E	Hematoxylin and eosin

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1 Introduction

Taurine (2-aminoethanesulphonic acid) is widely present in mammalian tissue and plays a role in many physiological processes, including development of the brain, osmoregulation, and cell membrane stabilization. It also has hypolipidemic effects, as well as anti-oxidant and hepatoprotective effects (Huxtable 1992). In particular, taurine supplementation inhibits hepatic lipid peroxidation and prevents membrane disintegration during hepatocarcinogenesis in rats (You and Chang 1997). Rats treated with β-alanine to induce taurine depletion were more susceptible to ethanol-induced hepatic dysfunction (Kerai et al. 2001).

Taurine has to be absorbed through its own transporter to have physiological activity. However, since antagonists such as β-alanine, β-aminobutyric acid, and gamma-aminobutyric acid have forms similar to that of taurine and compete with taurine for absorption into the cell, the amount of taurine absorbed is often limited. In order to overcome this disadvantage, numerous taurine derivatives were developed. There are many studies about taurine derivatives with various physiological functions. Taurine-chloramine and taurine-bromamine, formed by a reaction of taurine with HOCl and HOBr respectively, have anti-microbial and anti-inflammatory effects (Marcinkiewicz and Kontny 2014). Early taurine-chloramine supplementation *in vivo* delays the onset of collagen-induced arthritis (Marcinkiewicz et al. 1995). Furthermore, high doses of taurolidine significantly inhibited advanced intraperitoneal tumor growth in rats (Braumann et al. 2005). Thiotaurine, the thiosulfonate analog of taurine, protects against diabetes-related biochemical changes in the plasma, heart, and aorta (Budhram et al. 2013).

N-(D-Ribopyranosyl)taurine sodium salt (T-Rib), expected to be absorbed through a carbohydrate transporter, was a synthetic derived from taurine and ribose (Fig. 1). In addition, T-Rib was reported to inhibit adipocyte differentiation in human pre-adipocytes (Cho et al. 2014).

The purpose of this study was to investigate the effect of T-Rib supplementation on the hepatic antioxidant system in rat models of diet-induced obesity and taurine deficiency.

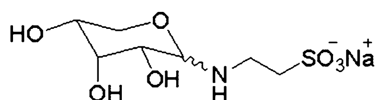

Fig. 1 Structure of T-Rib

2 Methods

2.1 Animals and Diet

Fifty-six male Sprague-Dawley rats aged 4 weeks were purchased from Koatech (Pyeongtaek, Korea). They were each housed in a cage in the laboratory animal room at Inha University under the following conditions: a constant 12-h light and dark cycle (8 AM to 8 PM), controlled temperature of 22 ± 2 °C, and 60 ± 5 % humidity. Food and water were provided *ad libitum*. The composition of the experimental diet was based on AIN 93G (Reeves et al. 1993) as shown in Table 1.

2.2 Experimental Design

The experimental design is shown in Fig. 2. Following 1 week of acclimatization with a pelletized commercial diet, the rats were randomly assigned to the following seven groups with eight animals in each group for a period of 6 weeks: N group, normal diet; HF group, high-fat diet; HFT group, high-fat diet and 4 mmol/kg/day taurine; HFA group, high-fat diet and β-alanine; HFR2 group, high-fat diet, β-alanine, and 2 mmol/kg/day T-Rib; HFR4 group, high-fat diet, β-alanine, and 4 mmol/kg/day T-Rib; and HFR6 group, high-fat diet, β-alanine, and 6 mmol/kg/day T-Rib.

The taurine deficiency rat model was induced through β-alanine supplementation in drinking water (3 % w/v). Taurine and T-Rib were orally administered to the

Table 1 Composition of experimental diet

	Experimental diets	
Ingredients	Normal diet (g)	High-fat diet (g)
Casein	200	200
Corn starch	529.486	105.036
Sucrose	100	232
Cellulose	50	50
Lard	0	175
Soybean oil	70	25
AIN-93 mineral mixture	35	35
AIN-93 vitamin mixture	10	10
DL-Methionine	3	3
Choline bitrartrate	2.5	2.5
Tert-butyl hydroquinone	0.014	0.014
Total	1,000	837.55

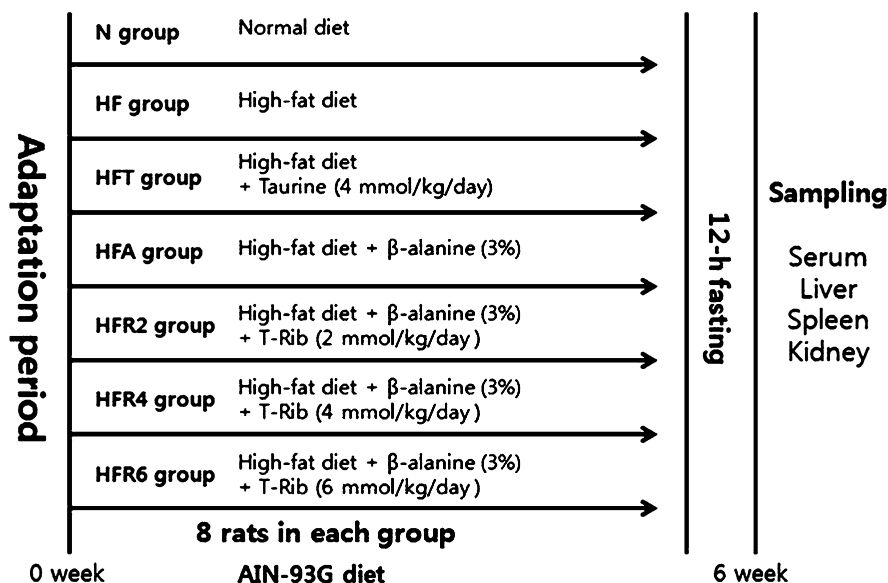

Fig. 2 Experimental design

HFT, HFR2, HFR4, and HFR6 groups, and the same amount of distilled water was orally administered to the N, HF, and HFA groups. Body weight (BW) was measured once per week.

2.3 *Sampling and Chemical Analysis*

After 6 weeks of treatment, the animals were deprived of food for 12 h and then sacrificed. Blood sample was collected by heart puncture, and the serum was separated by centrifugation at 1,650 × *g* for 20 min. The isolated serum was immediately frozen in liquid nitrogen, and then stored at −70 °C before analysis. The liver, spleen, and kidney were quickly removed, washed in saline, and then weighed.

To analyze thiobarbituric acid reactive substance (TBARS) and glutathione (GSH) content and glutathione peroxidase (GPx) activity, liver extracts were prepared. Approximately 4 g of minced liver tissue was mixed with 8 ml of cold potassium phosphate buffer (154 mM KCl, 50 mM Tris-HCl, 1 mM EDTA buffer, pH 7.4) in a homogenizer (POTTER S, B.BRAUN, Hessen, Germany). The homogenate was centrifuged for 10 min at 1,000 × *g* (VS 6000 CHN, Vision Scientific, Daejeon, Korea) to remove the precipitate. The above mentioned supernatant solution (1.2 ml) was stored for analyzing TBARS and GSH content, and the remaining supernatant solution was centrifuged for 20 min at 10,000 × *g* (Ananti J-25 centrifuge, Beckman Coulter, Inc., CA, USA) to remove the cell debris. The cytosol was obtained by centrifuging the supernatant solution by using an ultracentrifuge for

60 min at 100,000 × *g* to remove the microsome (Optima LE-80K Ultracentrifuge, Beckman Coulter, Inc., CA, USA). All procedures were carried out at 0–4 °C. After processing, all samples were immediately frozen in liquid nitrogen, and then stored at −70 °C until use.

Serum glutamine oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) activities were analyzed using an automatic analyzer (BPC BioSed srl, Italy). Standard serum (Asan Pharmaceutical, Korea) was used for calibration before every parameter was analyzed. All of the results were expressed as IU/L serum.

The hepatic lipid peroxide content was analyzed using thiobarbituric acid (TBA) in a method described by Ohkawa et al. (1979). In brief, 10 % of the homogenate was introduced into a falcon tube containing 1.5 ml of 1 % phosphoric acid and 0.5 ml of TBA reagent. After mixing, the tube was placed in a water bath for 45 min at 95 °C. The content was then cooled in an ice bath for 5 min in the dark, and then 2 ml of n-butanol was added. After mixing, centrifugation was performed for 15 min at 916 × *g*. The supernatant was measured at 535 nm by using the Powerwave X microplate spectrophotometer (Biotek Instruments Inc., VT, USA), and the difference was used to calculate the TBARS concentration, which was expressed as nmol/mg protein. The standard curve was prepared using 1,1,3,3-tetraethoxypropane.

Hepatic GSH content was measured according to the method described by Ellman (1959). Briefly, 0.1 ml of the supernatant was mixed with the reaction mixture, which contained 0.1 ml of 0.2 M Tris buffer (pH 8), 4 ml of methanol, and 0.1 ml of 0.01 M 5,5′-dithio-bis-2-nitrobenzoic acid. The solution was kept at room temperature for 15 min and then centrifuged for 30 min at 2,062 × *g*. The absorbance of the supernatant was measured at 415 nm by using the Powerwave X microplate spectrophotometer (Biotek Instruments Inc., Winooski, USA). The GSH contents were expressed as nmol/mg protein.

Hepatic GPx activity was measured according to a procedure described by Tappel (1977). Briefly, 0.1 ml of cytosol was mixed with 1.55 ml of reaction solution containing 50 mM Tris-HCl, 0.1 mM EDTA, 0.25 mM GSH, 0.12 mM NADPH, and 1 U/ml glutathione reductase. The solution was placed in a water bath for 4 min at 37 °C and then 0.05 ml of cumene hydroperoxide was added. The difference in absorbance between 0 and 1 min was measured at 340 nm by using a UV-visible spectrophotometer (DU 650, Beckman coulter, Inc., CA, USA). GPx activity was calculated by using the extinction coefficient of NADPH (0.00622 μM^{-1} cm^{-1}) and expressed as nmol NADPH oxidized/min/mg protein.

The protein content in the hepatic tissue was estimated according to the Lowry method (Lowry et al. 1951). Bovine serum albumin was used as a standard.

2.4 Histological Examination of Liver Tissue

Histological examination was performed on sections of liver tissue. The liver tissues were fixed immediately with 10 % buffered formalin after removal, and paraffin-embedded sections were stained with hematoxylin and eosin (H&E).

2.5 Statistical Analysis

All data were expressed as the means ± standard error (SE) using the SPSS 17.0 program. Data were analyzed for significant differences by one-way analysis of variation followed by Duncan's multiple range tests at $p < 0.05$.

3 Results and Discussion

3.1 Growth Curve and Major Organ Weights

Since a high fat diet is the main cause of obesity (Kopelman 2000), a rat model of high-fat diet-induced obesity was used. A rat model of β-alanine-induced taurine deficiency was also used. The body weights of the rats were monitored for 6 weeks. There was no difference among the body weights of the groups at the start of the experiment; however, at the end of the experimental period, HF and HFA groups showed significantly higher weights than those shown by the N, HFR4, and HFR6 groups (Table 2). In addition, the weight of the HFT group tended to be lower than that of the HF group, but the difference was not significant.

Administration of taurine inhibited obesity; this finding was similar to that of a previous study (Chang et al. 2010), but T-Rib inhibited obesity more effectively than taurine.

The weights of major organs including the liver, spleen, and kidney were measured and relative organ weights were calculated. There were no significant intergroup differences in the relative weights of the liver, spleen, and kidney (Fig. 3).

Table 2 Initial body weight and final body weight

Group	Initial body weight (g)	Final body weight (g)
N	188.3 ± 1.9[ns]	354.4 ± 12.7[a]
HF	187.3 ± 6.0	402.7 ± 6.6[b]
HFT	186.7 ± 6.4	384.6 ± 11.7[ab]
HFA	191.6 ± 5.9	405.3 ± 16.2[b]
HFR2	190.5 ± 4.6	370.5 ± 13.4[ab]
HFR4	190.2 ± 4.5	364.1 ± 5.9[a]
HFR6	187.0 ± 5.0	355.1 ± 6.1[a]

Values are expressed as means ± SE; *N* normal diet group, *HF* high-fat diet group, *HFT* high-fat diet + 4 mmol taurine group, *HFA* high-fat diet + β-alanine group, *HFR2* high-fat diet + β-alanine + 2 mmol T-Rib group, *HFR4* high-fat diet + β-alanine + 4 mmol T-Rib group, *HFR6* high-fat diet + β-alanine + 6 mmol T-Rib group. Values with different superscripts within the column are significantly different at $p < 0.05$ by Duncan's multiple range test, ns is not significant

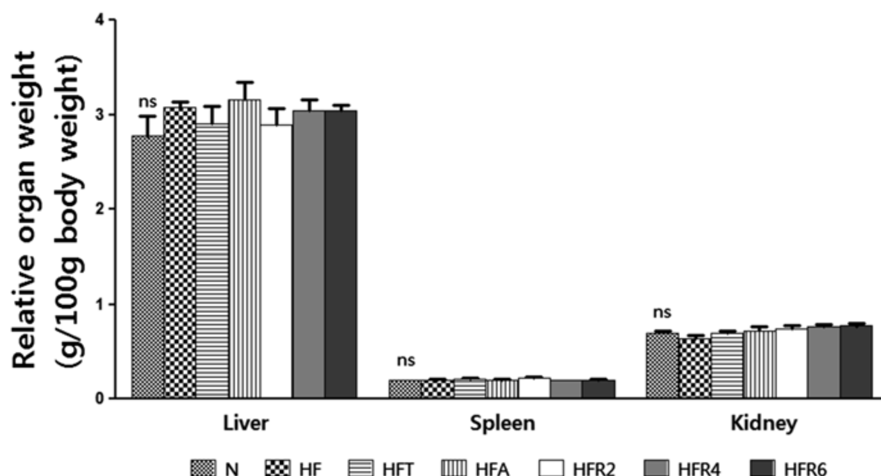

Fig. 3 Relative major organs weights. *N* normal diet group, *HF* high-fat diet group, *HFT* high-fat diet + 4 mmol/kg/day taurine group, *HFA* high-fat diet + 3 % β-alanine group, *HFR2* high-fat diet + 3 % β-alanine + 2 mmol/kg/day T-Rib group, *HFR4* high-fat diet + 3 % β-alanine + 4 mmol/kg/day T-Rib group, *HFR6* high-fat diet + 3 % β-alanine + 6 mmol/kg/day T-Rib group. Values are expressed as means ± SE; ns is not significant at $p < 0.05$, as determined by Duncan's multiple range test

3.2 Serum GOT and GPT Activities

Serum GOT and GPT activities are used as indicators of liver damage (such as fatty liver and hepatitis) since their activities increase in cases of liver damage (Angulo et al. 1999). The beneficial effects of taurine in decreasing GOT and GPT activity have been reported in rats (Chen et al. 2006) and obese children (Obinata et al. 1996) with non-alcoholic steatohepatitis.

There was no significant intergroup difference in serum GPT activity, but serum GOT activity tended to be higher in the HF and HFA groups than in the N group (Fig. 4). The taurine-administered groups showed significantly decreased serum GOT activity; in particular, the T-Rib-administered groups (HFR4 and HFR6) showed a dose-dependent decrease. These results suggest that taurine and T-Rib prevent liver damage.

3.3 Hepatic Antioxidant System

Since a high-fat diet is known to increase oxidative stress levels, which are also associated with cancer and aging, high-fat diets were reported to increase hepatic TBARS content, and decrease hepatic GSH content and GPx activity. In this study, a significant increase in hepatic TBARS content was observed in the HF group compared to that in the N group (Fig. 5). However, the taurine- and T-Rib-administered groups (HFR4 and HFR6) showed significantly smaller increases.

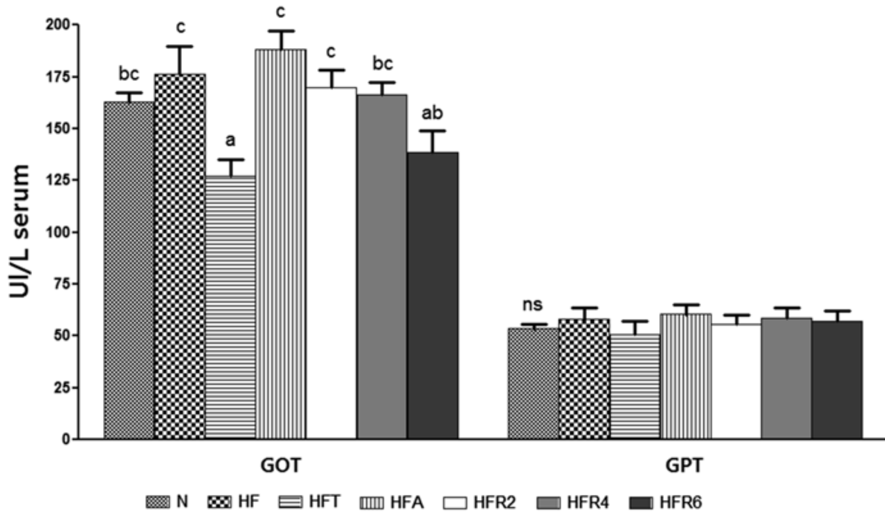

Fig. 4 Serum GOT and GPT activities. *N* normal diet group, *HF* high-fat diet group, *HFT* high-fat diet+4 mmol/kg/day taurine group, *HFA* high-fat diet+3 % β-alanine group, *HFR2* high-fat diet+3 % β-alanine+2 mmol/kg/day T-Rib group, *HFR4* high-fat diet+3 % β-alanine+4 mmol/kg/day T-Rib group, *HFR6* high-fat diet+3 % β-alanine+6 mmol/kg/day T-Rib group. Values are expressed as means ± SE; values with different superscripts are significantly different at $p < 0.05$, as determined by Duncan's multiple range test; ns is not significant

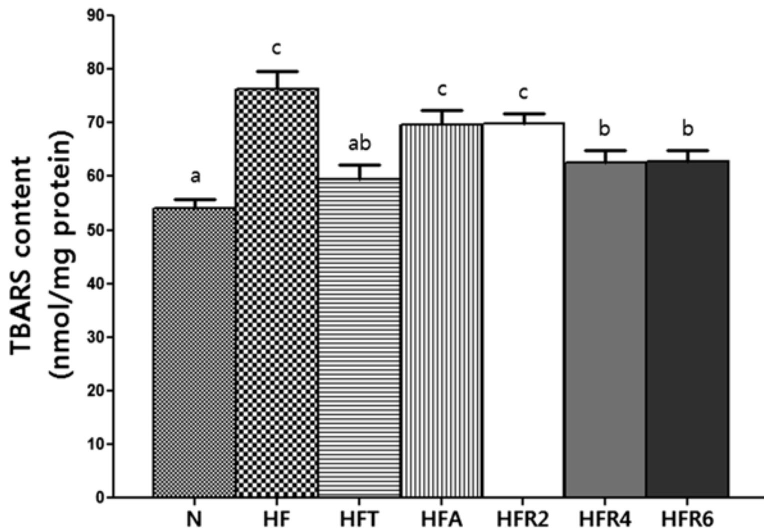

Fig. 5 Hepatic TBARS contents. *N* normal diet group, *HF* high-fat diet group, *HFT* high-fat diet+4 mmol taurine group, *HFA* high-fat diet+3 % β-alanine group, *HFR2* high-fat diet+3 % β-alanine+2 mmol T-Rib group, *HFR4* high-fat diet+3 % β-alanine+4 mmol T-Rib group, *HFR6* high-fat diet+3 % β-alanine+6 mmol T-Rib group. Values are expressed as means ± SE; values with different superscripts are significantly different at $p < 0.05$, as determined by Duncan's multiple range test

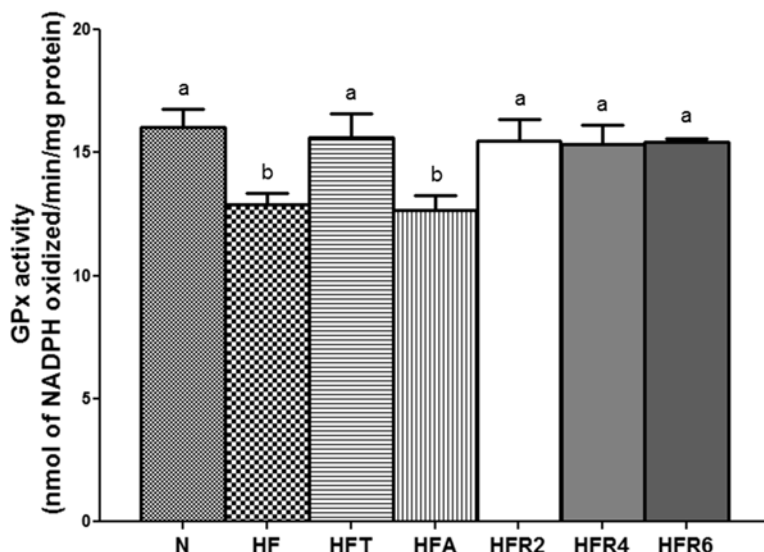

Fig. 6 Hepatic GPx activities. *N* normal diet group, *HF* high-fat diet group, *HFT* high-fat diet + 4 mmol/kg/day taurine group, *HFA* high-fat diet + 3 % β-alanine group, *HFR2* high-fat diet + 3 % β-alanine + 2 mmol/kg/day T-Rib group, *HFR4* high-fat diet + 3 % β-alanine + 4 mmol/kg/day T-Rib group, *HFR6* high-fat diet + 3 % β-alanine + 6 mmol/kg/day T-Rib group. Values with different superscripts are significantly different at $p < 0.05$, as determined by Duncan's multiple range test

GSH is an antioxidant in the body and is converted to GSSH (oxidative form) during oxidative stress (Franco et al. 2007). However, in this study, no significant differences were observed among the groups (data not shown).

It has been shown that obesity is related to a decrease in antioxidant capacity by lowering the activity of antioxidant enzymes in animal and human models. Hepatic GPx activity significantly decreased in the HF group and in the taurine- and T-Rib-administered groups (Fig. 6).

These results suggest that taurine and T-Rib is likely to play a role in the hepatic antioxidant system.

3.4 Histological Examination of Liver Tissue

It was reported that CCl_4-treated rats or high-fat diet-fed rats showed central vein disruption, ballooned lipid-laden hepatocytes, and dilated sinusoidal spaces (Desai et al. 2012). Liver tissue had a similar histological appearance in all groups (Fig. 7).

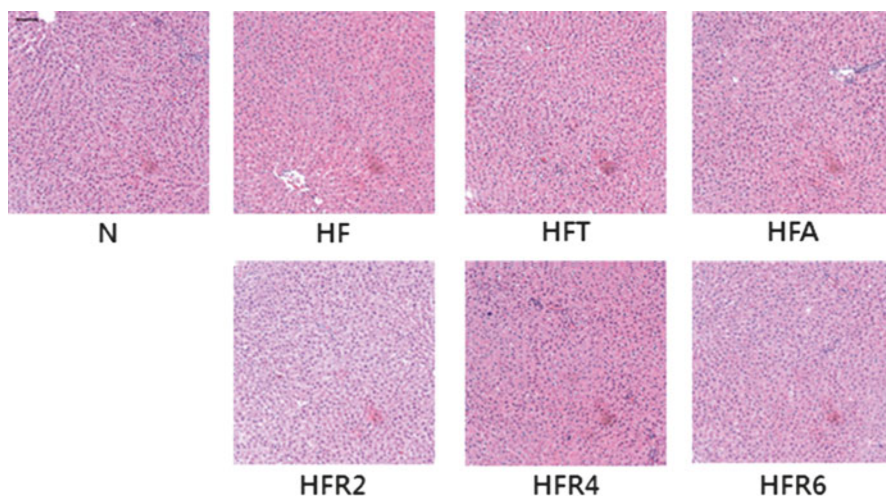

Fig. 7 Histological examination of liver tissue. Representative images of H&E stained sections of liver tissue from experimental groups. Scale bar = 100 μm. *N* normal diet group, *HF* high-fat diet group, *HFT* high-fat diet + 4 mmol/kg/day taurine group, *HFA* high-fat diet + 3 % β-alanine group, *HFR2* high-fat diet + 3 % β-alanine + 2 mmol/kg/day T-Rib group, *HFR4* high-fat diet + 3 % β-alanine + 4 mmol/kg/day T-Rib group, *HFR6* high-fat diet + 3 % β-alanine + 6 mmol/kg/day T-Rib group

4 Conclusion

It can be concluded from the present study that T-Rib as well as taurine supplementation seem to be beneficial to the hepatic antioxidant system in rat models of diet-induced obesity and taurine deficiency. In addition, T-Rib may be absorbed through a different transporter, not the normal taurine transporter. Further study is needed to investigate the mechanism of T-rib action.

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Evaluation of the Toxicity of a Single Oral Dose of *N*-(D-Ribopyranosyl)taurine Sodium Salt in Mice

Jeong Soon You, Yun Ju Lee, So Young Kim, Kyung Ja Chang, Hye Jeong Cho, and Sung Hoon Kim

Abbreviations

ANOVA	One-way analysis of variance
BW	Body weight
H&E	Hematoxylin and eosin
HFF	Health functional food
SEM	Standard error of the mean
T-Rib	*N*-(D-Ribopyranosyl)taurine sodium salt

1 Introduction

Taurine is a simple sulfur-containing compound and one of the most abundantly available free amino acids in human and animal tissues. Taurine has been reported to possess biological activities such as bile acid conjugation (Hardison 1978), physiological functions in the retina (Lombardini 1991), and both anti-oxidative (Keys and Zimmerman 1999) and anti-diabetic (Franconi et al. 1995) effects. However, taurine has some disadvantages such as poor absorption, high-dose requirement, and a fast renal extraction rate (Cho et al. 2014). To overcome these disadvantages, numerous taurine derivatives, such as thiotaurine (Budhram et al. 2013) taurine

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chloramine (Kontny et al. 2007), and tauromustine (Gunnarsson et al. 1989) have been developed.

Recent studies reported that taurine-carbohydrate derivatives possess anti-adipogenic effects on human adipocyte differentiation (Cho et al. 2014) with T-Rib showing hypolipidemic, anti-obesity (Kim et al. 2014a), and antioxidative (Kim et al. 2014b) effects in Sprague Dawley rats. In addition, a patent application has been filed for T-Rib in Korea (Kim et al. 2013).

Because of recent interest in health and in improving standards of living, the need for various HFFs is rising, forming a receptive world market for HFF. Health functional food is defined as "A product intended for use to enhance and preserve human health with one or more functional ingredients or constituents (Ministry of Food and Drug Safety 2010)". *N*-(D-Ribopyranosyl)taurine sodium salt with its various functions is likely to be developed as a HFF. In order for a compound to be developed as an ingredient of a HFF, the functionality and safety of the compound must be verified. Therefore, the objective of this study was to verify the safety of T-Rib by evaluating the toxicity of a single oral dose of T-Rib in ICR mice.

2 Methods

2.1 Animals and T-Rib

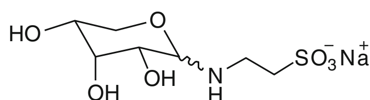
Male and female ICR mice were purchased from Koatech (Pyeongtaek, Korea). All mice were housed at the laboratory animal housing at Inha University according to the guidelines outlined by the Experimental Animal Ethics Committee and kept under a constant 12 h light and dark cycle (AM 09:00–PM 09:00) with controlled temperature (23 ± 1 °C) and humidity (55 ± 10 %).

N-(D-Ribopyranosyl)taurine sodium salt is a newly synthesized taurine-carbohydrate derivative from taurine and ribose. The reaction of D-ribose with taurine was carried out as described previously (Cho et al. 2014). The chemical structure of T-Rib was identified by nuclear magnetic resonance spectroscopy (Fig. 1).

The toxicity of a single oral dose of T-Rib was evaluated according to modified OECD-420 guidelines (OECD 1987). Eight ICR mice/group (four males and four females) were used for the experiment. Following 1 week of acclimatization, male and female ICR mice were randomly divided into three groups and fed a pelletized commercial diet for a period of 14 days. Feed and water were provided *ad libitum.*

Distilled water was administered to the control groups; the T-Rib treatment groups received either 2,000 mg T-Rib/kg BW (according to the OECD guidelines) or 5,000 mg T-Rib/kg BW (according to the harmless material classification standard of the US Environmental Protecting Agency). The respective T-Rib dose or control was administered via oral gavage with a 2 ml syringe at a volume of 20 ml/kg using distilled water.

Fig. 1 Structure of T-Rib



2.2 Cageside Observations

Cageside observations were performed every hour for 6 h and then every day for 14 days to evaluate the toxicity of T-Rib. Cageside observations included mortality and clinical signs such as changes in skin, fur, and eyes; respiratory and autonomic effects such as salivation, diarrhea, and urination; central nervous system effects including tremors, convulsions, relaxation, and coma (Demma et al. 2007). Body weights were measured on the day of administration (day 0) and both 7 and 14 days after T-Rib or control administration.

2.3 Sampling

At the end of the experimental period (14 days after T-Rib administration) and following 12 h of fasting, the animals were anesthetized with ether. A section of liver tissue was sampled for histological examination.

2.4 Histological Examination of Liver Tissue

Histological examination of liver tissue was performed using the paraffin method. Fresh tissues were fixed immediately with 10 % buffered formalin and paraffin-embedded sections were stained with hematoxylin and eosin (H&E). The stained sections were then examined by light microscopy (Axioskop 2, Zeiss, Jena, Germany).

2.5 Statistical Analysis

All analyses were performed using the SPSS 20.0 software program. Each value was expressed as the mean ± standard error of the mean (SEM). Changes in body and organ weights were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test at a $p < 0.05$.

3 Results and Discussion

3.1 *Cageside Observation of Mice*

All mice appeared to be healthy and normal throughout the experimental period and no mortalities were found in the experimental and control groups.

In addition, no abnormal clinical signs, such as changes in skin, fur, and eyes; respiratory and autonomic effects such as salivation, diarrhea, and urination; and central nervous system effects, including tremors, convulsions, relaxation, and coma were observed regardless of administration.

3.2 *Body Weight Measurement of Mice*

No significant differences in BW were found between the experimental groups from day 0 to day 14 (Table 1). It has previously been reported (Lee et al. 2007, 2012) that BW decreased transiently after acute toxicity test of 5,000 mg/kg BW of Leuconostoc eitreum GJ7 or *Lactobacillus plantarum* AF1 isolated from kimchi. However, BW returned to normal levels after a relatively short period. Based on our findings from this study that no difference in body weight was found 7 days after administration of T-Rib, a single oral dose of T-Rib may have no negative effect on the growth of mice in the experimental groups compared with the control group.

3.3 *Histological Examination of Liver Tissue*

Previous histological examinations have shown that *in vivo* administration of the toxic compounds, t-BHP and azathioprine, causes neutrophil infiltration, swelling of hepatocytes, and liver necrosis (Wang et al. 2000; Amin and Hamza 2005).

In our study, histological examination of liver tissue revealed no significant differences between the experimental groups and the control group (Figs. 2 and 3).

Table 1 Body weight of mice

Sex	T-Rib (mg/kg BW)	Number of mice	Days after treatment		
			Day 0	Day 7	Day 14
Male	0	4	32.8 ± 0.7[a]	35.8 ± 0.6	36.5 ± 0.7[ns]
	2,000	4	32.8 ± 0.6	35.1 ± 0.9	36.5 ± 0.8
	5,000	4	32.8 ± 0.6	35.0 ± 1.0	37.0 ± 1.0
Female	0	4	26.9 ± 0.5	27.5 ± 0.8	28.0 ± 0.5[ns]
	2,000	4	26.7 ± 0.4	27.8 ± 0.5	28.4 ± 0.9
	5,000	4	26.9 ± 0.4	28.3 ± 0.2	29.2 ± 0.5

[a]Values are expressed as mean ± SEM; *ns* not significant

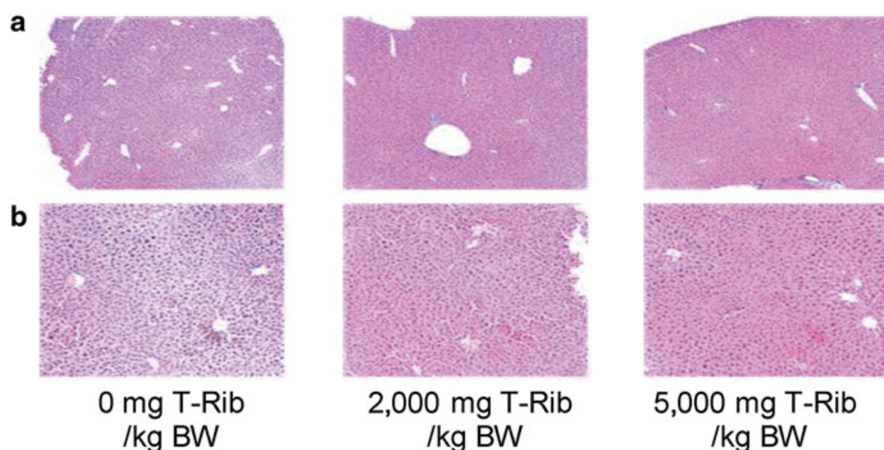


Fig. 2 Light micrography of hepatocytes in male mice. (**a**) 100× magnification; (**b**) 200× magnification. Representative pictures are H&E-stained liver sections. No significant differences were found in liver histology between the different treatment groups

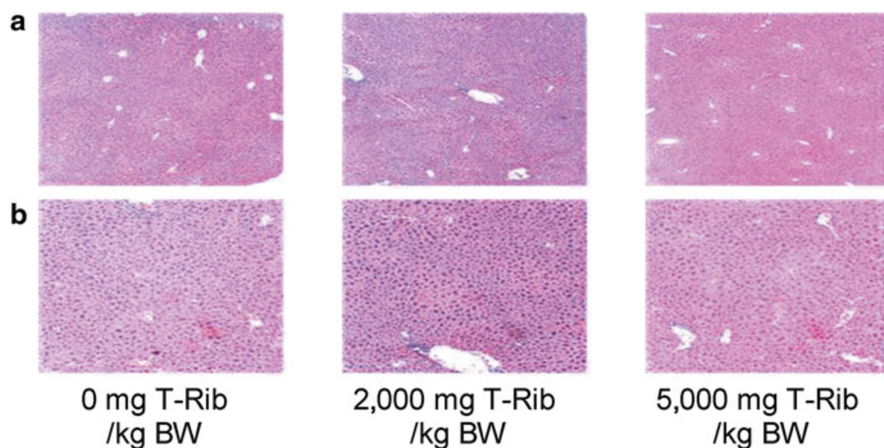


Fig. 3 Light micrography of hepatocytes in female mice. (**a**) 100× magnification; (**b**) 200× magnification. Representative pictures are H&E-stained liver sections. No significant differences were found in liver histology between the different treatment groups

4 Conclusions

In this study, we evaluated the toxicity of a single oral dose of T-Rib in male and female ICR mice to verify its safety as a potential ingredient of a HFF. Our results showed no mortality, abnormal clinical signs, and differences in body weight in all treatment groups. In addition, no abnormal differences were observed in the

histological examination of liver tissue of T-Rib-treated and control mice. We therefore suggest that a single oral dose of T-Rib as high as 5,000 mg/kg BW exerts no toxic response in mice. However, further studies assessing the toxicity of repetitive oral dosing are required to define the safety profile of T-Rib for future HFF use.

Acknowledgements We thank Dong-A Pharmaceutical Co. for the donation of taurine.

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N-(*β*-D-Xylopyranosyl)taurine Sodium Salt Supplementation Has Beneficial Effect on the Hepatic Antioxidant System of Rats Fed a High-Fat Diet and β-Alanine

Jeong Soon You, Yun Ju Lee, So Young Kim, Kyung Ja Chang, Hye Jeong Cho, and Sung Hoon Kim

Abbreviations

T-Xyl	*N*-(*β*-D-Xylopyranosyl)taurine sodium salt
N	Normal diet
HF	High-fat diet
HFT	High-fat diet + 4 mmol/kg/day taurine
HFA	High-fat diet + 3 % β-alanine
HFTX2	High-fat diet + 3 % β-alanine + 2 mmol/kg/day T-Xyl
HFTX4	High-fat diet + 3 % β-alanine + 4 mmol/kg/day T-Xyl
HFTX6	High-fat diet + 3 % β-alanine + 6 mmol/kg/day T-Xyl
BW	Body weight
GOT	Glutamate oxaloacetate transaminase
GPT	Glutamate pyruvate transaminase
TBARS	Thiobarbituric acid reactive substances
GSH	Glutathione
GPx	Glutathione peroxidase
H&E	Hematoxylin and eosin
SEM	Standard error of mean

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1 Introduction

The consumption of a high-fat diet or a high-calorie diet induces oxidative stress and inflammation, which may be the cause of various diseases such as cancer, stroke, Parkinson's disease, and atherosclerosis (Ashok and Ali 1999). Many researchers are interested in studying the antioxidant system for the prevention and treatment of these diseases.

Although taurine exhibits antioxidant activity, the pharmacokinetics of taurine are not very favorable showing disadvantages such as a high dose requirement and poor absorption (Cho et al. 2014). Recent studies reported the synthesis of numerous taurine derivatives such as taurine chloramine (Janusz et al. 1995), taurine bromamine (Janusz 2009), and taurine carbohydrates (Cho et al. 2014) whose functions were also verified. T-Xyl, one of the carbohydrate derivatives of taurine, has been reported to possess an anti-adipogenic effect *in vitro* and a patent application for it has been filed in Korea (Kim et al. 2013).

β-Alanine is a structural analog of taurine, and thus, they act competitively with each other. Therefore, administration of β-alanine induces taurine depletion. Studies have reported β-alanine mediated retinal damage in cats (Sturman et al. 1996) and an increase in serum total cholesterol levels and low-density lipoprotein cholesterol levels in rats with diabetes (Chang 1999).

The objective of this study was to test the hypothesis that T-Xyl exerts positive effects on the hepatic antioxidant system of rats fed a high-fat diet and β-alanine. In order to test this hypothesis, we induced oxidative stress by feeding rats a high-fat diet. Furthermore, to determine whether T-Xyl was absorbed through the taurine transporter system, we administered β-alanine to the rats. The activities of glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) in serum, hepatic thiobarbituric acid reactive substances (TBARS) and glutathione (GSH) contents, and cytosolic glutathione peroxidase (GPx) activity were also measured to determine the effect of T-Xyl on the hepatic antioxidant system. Furthermore, we examined hepatic histology of rats fed a high-fat diet and β-alanine.

2 Materials and Methods

2.1 Preparation of T-Xyl and Animals

N-(β-D-Xylopyranosyl)taurine sodium salt was newly synthesized from taurine and xylose as described previously and was identified using nuclear magnetic resonance (Cho et al. 2014).

Male Sprague-Dawley rats (4-week-old) were purchased from Koatech (Pyeongtaek, Korea). In accordance with the guidelines outlined by the Experimental Animal Ethics Committee of Inha University, all rats were kept in laboratory animal housing at Inha University, and the procedures of rat care were conducted with a

Table 1 Composition of experimental diet (g/100 g diet)

	Experimental diets	
Component	Normal diet (g)	High-fat diet (g)
Casein	200	200
Corn starch	529.486	105.036
Sucrose	100	232
Cellulose	50	50
Lard	0	175
Soybean oil	70	25
AIN-93 Mineral mixture	35	35
AIN-93 Vitamin mixture	10	10
DL-Methionine	3	3
Choline bitrartrate	2.5	2.5
Tert-butyl hydroquinone	0.014	0.014
Total	1,000	837.55

constant 12-h light and dark cycle (09:00 a.m. to 09:00 p.m.), controlled temperature (22 ± 2 °C), and humidity (55 ± 5 %). The composition of the experimental diet was based on AIN-93 (Reeves et al. 1993) as shown in Table 1. Diet and water were provided *ad libitum.*

After 1 week of acclimatization with a pelletized commercial diet, the rats were randomly divided into the following seven groups with eight rats in each group: N, normal diet; HF, high-fat diet; HFT, high-fat diet + 4 mmol/kg/day taurine; HFA, high-fat diet + 3 % β-alanine; HFTX2, high-fat diet + 3 % β-alanine + 2 mmol/kg/day T-Xyl; HFTX4, high-fat diet + 3 % β-alanine + 4 mmol/kg/day T-Xyl; HFTX6, high-fat diet + 3 % β-alanine + 6 mmol/kg/day T-Xyl. Taurine and T-Xyl were administered orally to the rats in the HFT and HFTXs groups and the same amount of distilled water was administered orally to the rats in the N, HF and HFA groups. β-Alanine was supplemented by dissolving in feed water (3 % w/v). Food and water intake was measured twice per week and body weight was measured once per week.

2.2 *Sampling and Tissue Preparation*

At the end of the experimental period (after 6 weeks), the rats were fasted for 12 h and subsequently, anesthetized with ether before being sacrificed. Blood was collected from the heart. The liver, kidney, and spleen were weighed and their weights were calculated relative to body weight (BW). A portion of the liver was removed from the rats for histological photographs and for the preparation of liver extract.

For preparation of liver extract, approximately 3 g of minced liver tissue was homogenized with 6 ml of ice-cold homogenizing media (154 mM KCL, 50 mM Tris-Hcl, and 1 mM EDTA buffer; pH 7.4) by using a homogenizer at 4 °C. The homogenate was centrifuged using a centrifuge (VS-6000, Vision, Korea) for

10 min at 1,000 × *g* for removal of the precipitate after which 1.2 ml of the above-mentioned supernatant solution was stored for analysis of TBARS and GSH contents. The remaining supernatant solution was centrifuged for 20 min at 10,000 × *g* for removal of cell debris. Cytosol was obtained by centrifuging the supernatant solution for 60 min at 100,000 × *g* by using an ultracentrifuge (Beckman Coulter, Inc., Fullerton, CA). The serum was obtained by centrifugation at 1,650 × *g* for 20 min. All serum and samples were immediately frozen in liquid nitrogen, and then stored in a deep freezer maintained at −70 °C until analysis.

2.3 Biochemical Analysis

GOT and GPT activities in serum were analyzed using an automatic analyzer (BPC BioSed srl, Rome, Italy).

Hepatic lipid peroxide content was analyzed using the method of thiobarbituric acid (TBA) described by Buege (1978). The standard curve was prepared using 1,1,3,3-tetramethoxypropane and TBARS concentration was expressed as nmol/mg protein.

Analysis of hepatic GSH content was performed using the method of Ellman (1959) with modification. In brief, 100 μL of liver homogenate was mixed with the reaction mixture, which contained 0.1 ml of 0.2 M Tris-buffer (pH 8), 4 ml of methanol, and 0.1 ml of 0.01 M 5,5′-duthiobis-2-nitrobenzoic acid. The solution was kept at room temperature for 15 min and then centrifuged for 30 min at 2,062 × *g* (VS-6000, Vision, Korea). The supernatant was measured at 415 nm by using a Powerwave X microplate spectrophotometer (Biotek Instruments Inc., Winooski, USA). GSH content was expressed as nmol/mg protein.

GPx activity was analyzed using the method described by Tappel (1977). Cumene hydroperoxide was used as the peroxide substrate in this method. The decrease in absorbance of NADPH was measured at 340 nm by using a spectrometer (DU-650, Beckman, Schaumburg, IL). One unit defined as 1 nmol of NADPH was converted to $NADP^+$ per minute at 37 °C and pH 7.6. The results of GPx activity were expressed as IU per mg protein.

2.4 Histological Photograph of the Liver

A histological photograph of liver tissue was examined under a light microscope (Axioskop 2, Zeiss, Jena, Germany) by using the paraffin method. Fresh tissues were fixed immediately with 10 % buffered formalin, and paraffin-embedded sections were stained with hematoxylin and eosin (H&E).

2.5 Statistical Analysis

Data were expressed as means ± standard error of the mean (SEM). These data were used in one-way analysis of variance followed by Duncan's multiple range tests at $p<0.05$ for analysis for significant difference. All analyses were performed using SPSS 20.0 program.

3 Results and Discussion

3.1 The Effect of T-Xyl on Body Weight and Organ Weight

At the end of the experiment, the body weight gain in the HF group and HFA groups was higher than that in the N group. However, the body weight gain in the HFTXs group was not as high as that in the HF and HFA groups ($p<0.05$). The body weight gain in the HFT group was also lower than that in the HF group, but this difference was not significant. These results indicate that T-Xyl suppresses body weight gain more effectively than taurine.

In this study, no significant difference in diet intake was observed between the N and HF groups. However, the diet intake of the HFA group tended to be higher but not significantly different from those of the other groups except for the HFTX6 group (Table 2). It has been reported that administration of 1.5 % taurine to rats did not affect the intake of diet (Choi et al. 2006).

In addition, no significant differences were observed in the relative weights of the liver, spleen, and kidney among the groups (data were not shown).

Table 2 The effect of T-Xyl on body weight gain, diet intake, and water intake

Group	Body weight gain (g/6 weeks)	Diet intake (g/day)	Water intake (ml/day)
N	161.3 ± 10.3^{a}	20.8 ± 1.3^{ab}	25.4 ± 1.3^{a}
HF	210.6 ± 4.4^{c}	18.2 ± 0.7^{ab}	30.3 ± 3.4^{ab}
HFT	192.7 ± 9.2^{bc}	21.5 ± 1.7^{ab}	33.5 ± 1.9^{b}
HFA	213.2 ± 10.9^{c}	21.9 ± 1.5^{b}	31.2 ± 2.6^{ab}
HFTX2	180.4 ± 6.1^{ab}	18.5 ± 0.9^{ab}	30.6 ± 1.9^{ab}
HFTX4	177.5 ± 6.2^{ab}	18.7 ± 0.9^{ab}	35.8 ± 2.4^{b}
HFTX6	174.6 ± 7.9^{ab}	17.6 ± 1.3^{a}	32.7 ± 2.0^{ab}

Values are expressed as means ± SEM. *N* normal diet, *HF* high-fat diet, *HFT* high-fat diet + 4 mmol/kg/day taurine, *HFA* high-fat diet + 3 % β-alanine, *HFTX2* high-fat diet + 3 % β-alanine + 2 mmol/kg/day T-Xyl, *HFTX4* high-fat diet + 3 % β-alanine + 4 mmol/kg/day T-Xyl, *HFTX6* high-fat diet + 3 % β-alanine + 6 mmol/kg/day T-Xyl. Values with different superscript indicators are significantly different at $p<0.05$ by Duncan's multiple range tests

Table 3 The effect of T-Xyl on activities of serum GOT and GPT

Group	GOT (IU/L)	GPT (IU/L)
N	172.4 ± 4.1[bc]	53.4 ± 2.3[a]
HF	187.9 ± 14.2[cd]	90.5 ± 8.6[c]
HFT	129.9 ± 12.5[a]	50.8 ± 6.2[a]
HFA	191.6 ± 7.9[d]	75.7 ± 6.4[bc]
HFTX2	157.9 ± 8.7[bc]	63.4 ± 6.1[ab]
HFTX4	153.9 ± 5.2[ab]	61.9 ± 6.9[ab]
HFTX6	159.9 ± 9.5[bc]	66.1 ± 5.4[ab]

Values are expressed as means ± SEM. *N* normal diet, *HF* high-fat diet, *HFT* high-fat diet + 4 mmol/kg/day taurine, *HFA* high-fat diet + 3 % β-alanine, *HFTX2* high-fat diet + 3 % β-alanine + 2 mmol/kg/day T-Xyl, *HFTX4* high-fat diet + 3 % β-alanine + 4 mmol/kg/day T-Xyl, *HFTX6* high-fat diet + 3 % β-alanine + 6 mmol/kg/day T-Xyl. Values with different superscript indicators are significantly different at $p < 0.05$ by Duncan's multiple range tests

3.2 The Effect of T-Xyl on Activities of Serum GOT and GPT

Results for activities of GOT and GPT in serum are shown in Table 3. The activity of serum GOT tended to be higher in the HF and HFA groups than in the N group, and significantly decreased in the HFT group. Compared to the HFA group, the HFTX groups showed a significant decrease in the activity of serum GOT.

Compared to the N group, the HF group showed a significant increase in the activity of serum GPT. In contrast, taurine or T-Xyl administration caused a significant decrease in serum GPT activity compared to that in the HF group. GOT and GPT exist in hepatocytes and are released into serum during hepatic damage or necrosis, which is an indicator of hepatic damage (Takeda et al. 1964). Therefore, these results suggest that taurine or T-Xyl prevents hepatic damage.

3.3 The Effect of T-Xyl on Hepatic TBARS and GSH Contents

Results for hepatic TBARS and GSH content are shown in Table 4. Compared to the N group, the HF and HFA groups showed a significant increase in hepatic TBARS content. In contrast, administration of taurine and T-Xyl caused a significant decrease in TBARS content compared to that in the HF group. The results indicate that a high-fat diet increased oxidative stress and that administration of taurine or T-Xyl resulted in inhibition of oxidative stress in rats fed a high-fat diet. Hepatic GSH is used as a substrate of the hepatic antioxidant defense system against oxidative stress (Hayes and McLellan 1999). However, in this study, no significant difference was observed in the hepatic GSH content among all groups.

Table 4 The effect of T-Xyl on hepatic TBARS and GSH contents

Group	TBARS content (nmol/mg protein)	GSH content (mnol/mg protein)
N	55.3 ± 2.0[ab]	113.2 ± 3.2[ns]
HF	79.8 ± 3.3[d]	106.3 ± 1.9
HFT	59.5 ± 2.7[bc]	108.6 ± 2.1
HFA	65.0 ± 2.7[c]	105.7 ± 1.5
HFTX2	56.6 ± 2.4[ab]	107.5 ± 2.4
HFTX4	59.4 ± 2.7[bc]	107.6 ± 2.7
HFTX6	50.9 ± 2.5[a]	109.0 ± 1.3

Values are means ± SEM. *N* normal diet, *HF* high-fat diet, *HFT* high-fat diet + 4 mmol/kg/day taurine, *HFA* high-fat diet + 3 % β-alanine, *HFTX2* high-fat diet + 3 % β-alanine + 2 mmol/kg/day T-Xyl, *HFTX4* high-fat diet + 3 % β-alanine + 4 mmol/kg/day T-Xyl, *HFTX6* high-fat diet + 3 % β-alanine + 6 mmol/kg/day T-Xyl. Values with different superscript indicators are significantly different at $p < 0.05$ by Duncan's multiple range tests, *ns* not significant

Oxidative stress is associated with various diseases, such as cancer, cardiovascular disease, diabetes mellitus, and hypertension (Giugliano et al. 1995). Therefore, T-Xyl could be used for the prevention or treatment of these diseases.

3.4 The Effect of T-Xyl on Cytosolic GPx Activity

GPx, a selenium-containing enzyme, oxidizes reduced glutathione (GSH) to oxidized glutathione (GSSG) and in the process catalyzes the reduction of either H_2O_2 or lipid peroxide. Thus, it is the first line of defense for the system against oxidative stress (Charde et al. 2011).

Results for cytosolic GPx activity are shown in Table 5. Cytosolic GPx activity was lower in the HF and HFA groups than in the N group. Administration of taurine and T-Xyl resulted in a significant increase in cytosolic GPx activity. Thus, these results indicate that a high-fat diet decreased the activity of the antioxidant enzyme and that administration of taurine or T-Xyl resulted in an increase in the GPx activity in rats fed a high-fat diet.

3.5 The Effect of T-Xyl on the Histological Photograph of the Liver

It has been reported that the HF group shows noticeable fatty liver while administration of lotus root and taurine suppresses formation of lipid droplets (Du et al. 2010). However, in this study, hepatocytes in the all groups had a similar histological appearance (Fig. 1).

Table 5 The effect of T-Xyl on cytosolic GPx activity

Group	GPx activity (nmol NADPH/min/mg protein)
N	16.0 ± 0.8[c]
HF	12.6 ± 0.4[ab]
HFT	14.9 ± 0.9[c]
HFA	12.1 ± 0.7[a]
HFTX2	14.0 ± 0.3[abc]
HFTX4	14.2 ± 0.6[bc]
HFTX6	14.2 ± 0.1[bc]

Values are expressed as means ± SEM. *N* normal diet, *HF* high-fat diet, *HFT* high-fat diet + 4 mmol/kg/day taurine, *HFA* high-fat diet + 3 % β-alanine, *HFTX2* high-fat diet + 3 % β-alanine + 2 mmol/kg/day T-Xyl, *HFTX4* high-fat diet + 3 % β-alanine + 4 mmol/kg/day T-Xyl, *HFTX6* high-fat diet + 3 % β-alanine + 6 mmol/kg/day T-Xyl. Values with different superscript indicators are significantly different at $p < 0.05$ by Duncan's multiple range tests

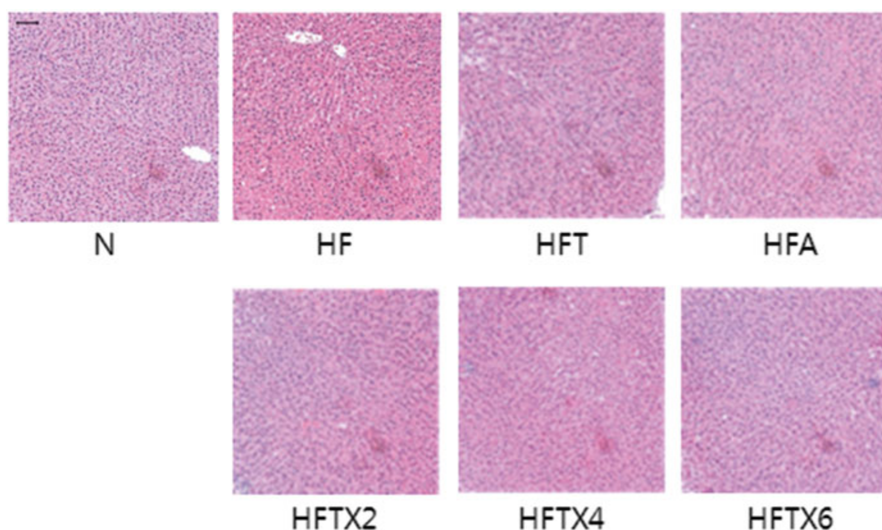

Fig. 1 The effect of T-Xyl on the histological photograph of liver. *N* normal diet, *HF* high-fat diet, *HFT* high-fat diet + 4 mmol/kg/day taurine, *HFA* high-fat diet + 3 % β-alanine, *HFTX2* high-fat diet + 3 % β-alanine + 2 mmol/kg/day T-Xyl, *HFTX4* high-fat diet + 3 % β-alanine + 4 mmol/kg/day T-Xyl, *HFTX6* high-fat diet + 3 % β-alanine + 6 mmol/kg/day T-Xyl. Representative pictures of H&E-stained sections of the liver from rats of all are not shown lipid drop accumulations and liver toxicity (200× magnification), Scale bar: 100 μm

4 Conclusion

The results of our study show that T-Xyl has a positive effect on the hepatic antioxidant system in rats fed a high-fat diet and β-alanine. It was also noted that when the rats were administered T-Xyl with β-alanine, T-Xyl was not absorbed through the same transporter as taurine. This observation suggests that T-Xyl may be absorbed through other transporter systems (for instance a carbohydrate transporter). Further study is needed to verify the mechanism of action of T-Xyl.

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A Safety Assessment of *N*-(β-D-Xylopyranosyl) Taurine Sodium Salt in Male and Female ICR Mice

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Abbreviations

ANOVA	One-way analysis of variance
BW	Body weight
H&E	Hematoxylin and eosin
HFF	Health functional food
LD_{50}	Median lethal dose
SEM	Standard error of the mean
SPSS	Statistical Product and Service Solution
T-Xyl	*N*-(*β*-D-Xylopyranosyl)taurine sodium salt

1 Introduction

Taurine has many biological benefits, including metabolic syndrome prevention (Kim et al. 2010), antioxidative (Keys and Zimmerman 1999), anti-obesity (Tsuboyama-Kasaoka et al. 2006; Kim et al. 2010), and anti-diabetic effects (Cheong and Chang 2013). However, because of various disadvantages of taurine, taurine derivatives were developed and their functions have been verified (Kontny et al. 2007; Budhram et al. 2013). Recently, taurine-carbohydrate derivatives were also developed, and T-Xyl, one such taurine-carbohydrate derivative, exhibited good anti-adipogenic effect *in vitro* (Cho et al. 2014) and anti-obesity effect *in vivo*

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(Kim et al. 2014). Therefore, it seems that T-Xyl has the potential to be developed as a specific ingredient of health functional food (HFF).

Due to its fast renal extraction rate, taurine is considered a non-toxic ingredient. However, it has been reported that rats supplemented with 5 % taurine have diarrhea. For the clinical use of T-Xyl in the future, it is very important that we first assess its safety.

Therefore, the objective of this study was to perform a safety assessment of T-Xyl. To evaluate the safety of T-Xyl, we conducted the acute oral toxicity test and determined the LD_{50} of T-Xyl in male and female ICR mice.

2 Methods

2.1 Animals and T-Xyl

ICR mice of both sexes were supplied from Koatech (Pyeongtaek, Korea). All mice were housed at the laboratory animal housing at Inha University and the procedures for mice care were conducted in accordance with the guidelines outlined by the Experimental Animal Ethics Committee of Inha University with a constant 12-h light and dark cycle (09:00 AM to 09:00 PM), controlled temperature (23 ± 1 °C), and controlled humidity (55 ± 10 %). All mice were fed a commercial pellet diet supplied by DBL (Anseong, Korea) and were provided water *ad libitum*.

N-(*β*-D-Xylopyranosyl)taurine sodium salt (Fig. 1) was synthesized recently with the goal of enhancing the absorption rate and improving the liposolubility and physiological activities in comparison with taurine (Cho et al. 2014).

2.2 The Toxicity Test of Acute Oral Dose

To assess the safety of T-Xyl, we conducted the acute oral toxicity test and determined the LD_{50} of T-Xyl in male and female ICR mice. The acute oral toxicity test of T-Xyl followed modified OECD-420 guidelines (OECD 1987).

Following 1 week of acclimatization, 12 ICR mice of each sex were randomly assigned to three groups of 4 mice each and fed for 14 days. Mice in each group were administered T-Xyl via oral gavage at a single dose of 0 (control), 2,000

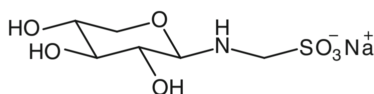

Fig. 1 Structure of T-Xyl. The chemical structure of T-Xyl was identified by nuclear magnetic resonance spectroscopy

(in accordance with OECD guidelines), or 5,000 (in accordance with the harmless material classification standard of the US Environmental Protection Agency) mg T-Xyl/kg BW. The administration volume was 2 ml/100 g BW and distilled water was administered to the control groups.

In addition, we determined the LD_{50} of T-Xyl. LD_{50} is the amount of a toxin, given all at once, which causes the death of 50 % of the group of test animals.

2.3 Body Weight Measurement and Clinical Signs Observation

Individual body weights were measured and recorded prior to administration of T-Xyl and at 7 and 14 days following T-Xyl or control administration.

Observations for clinical signs were made every hour for the first 6 h and then once daily for 14 days. Clinical signs included changes in skin, fur, and eyes; respiratory effects; autonomic effects, including salivation, diarrhea, and urination; and central nervous system effects, including tremors, convulsions, relaxation, and coma (Demma et al. 2007).

2.4 Sampling and Organ Weight Measurement

At the end of the observation period, the mice were fasted for 12 h before sacrifice. All mice were subject to macroscopic examination. The absolute organ weights of the liver, kidneys, and spleen were determined and the relative organ weights (g/100 g BW) were calculated. Sections of liver tissue were removed for histological evaluation.

2.5 Histological Examination of Liver Tissue

Histological examination was performed on sections of liver tissue under a light microscope (Axioskop 2, Zeiss, Jena, Germany) using the paraffin method. The liver tissues were fixed immediately with 10 % buffered formalin after removal, and paraffin-embedded sections were stained with hematoxylin and eosin (H&E).

2.6 Statistical Analysis

Data are expressed as means ± standard error of the mean (SEM). Statistical evaluations were performed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test at $p < 0.05$ for analysis of significant differences. All analyses were performed using the Statistical Product and Service Solution (SPSS) 20.0 software program.

3 Results and Discussion

3.1 *Mortality and* LD_{50}

Among all groups, there was no animal death prior to scheduled sacrifice. Therefore, final mortality was 0 % in all experimental groups.

According to the conventional LD_{50} tests (Lipnick et al. 1995), generally females are slightly more sensitive than males in their acute toxicity response. Therefore, females are normally used, but we used both sexes. In this study, the LD_{50} value of T-Xyl was >5,000 mg/kg BW in both male and female ICR mice.

3.2 *Body Weight Measurement and Clinical Signs Observation*

The body weight measurements are shown in Table 1. From these results, single-dose administration of T-Xyl had no effect on BW of groups administered T-Xyl compared to control groups. There was no significant difference in the BW from day 0 to day 14 in male or female mice among all groups.

Following single-dose administration, there were no abnormal clinical signs such as changes in skin, fur, or eyes; respiratory effects; autonomic effects, including salivation, diarrhea, and urination; or central nervous system effects, including tremors, convulsions, relaxation, and coma in all groups administered T-Xyl.

3.3 *Organ Weight Measurement*

At the end of observation period, all animals were sacrificed and major organs were subject to macroscopic examination. No abnormal findings or lesions were found in the liver, spleen, lung, heart, stomach, and kidney of all animals.

Table 1 Body weight of male and female ICR mice

			Days after treatment		
Sex	T-Xyl (mg/kg BW)	Number of mice	Day 0 (g)	Day 7 (g)	Day 14 (g)
Male	0	4	32.8 ± 0.7[a]	35.8 ± 0.6	36.5 ± 0.7[ns]
	2,000	4	32.8 ± 0.5	34.6 ± 0.9	36.6 ± 0.9
	5,000	4	32.9 ± 0.5	35.7 ± 0.7	37.1 ± 0.7
Female	0	4	26.9 ± 0.5	27.5 ± 0.8	28.0 ± 0.5
	2,000	4	26.7 ± 0.4	27.4 ± 0.7	28.9 ± 0.5
	5,000	4	26.9 ± 0.4	27.6 ± 0.9	28.1 ± 0.7

[a]Values are expressed as means ± SEM; *ns* not significant

Table 2 Organ weights of male and female ICR mice

Sex	T-Xyl (mg/kg BW)	Number of mice	Liver (g)	Spleen (g)	Lung (g)	Kidney (g)
Male	0	4	1.5 ± 0.06[a]	0.1 ± 0.02	0.2 ± 0.01	0.5 ± 0.03[ns]
	2,000	4	1.5 ± 0.07	0.1 ± 0.01	0.2 ± 0.01	0.5 ± 0.03
	5,000	4	1.5 ± 0.07	0.1 ± 0.01	0.2 ± 0.01	0.5 ± 0.02
Female	0	4	1.1 ± 0.10	0.1 ± 0.02	0.2 ± 0.01	0.3 ± 0.02[ns]
	2,000	4	1.2 ± 0.07	0.1 ± 0.00	0.2 ± 0.01	0.3 ± 0.01
	5,000	4	1.1 ± 0.07	0.1 ± 0.01	0.2 ± 0.00	0.3 ± 0.03

[a]Values are expressed as means ± SEM; *ns* not significant

Table 3 Relative organ weights of male and female ICR mice

Sex	T-Xyl (mg/kg BW)	Number of mice	Liver (g)	Spleen (g)	Lung (g)	Kidney (g)
			g/100 g BW			
Male	0	4	4.2 ± 0.1[a]	0.3 ± 0.04	0.6 ± 0.02	1.4 ± 0.06[ns]
	2,000	4	4.3 ± 0.2	0.3 ± 0.03	0.6 ± 0.02	1.4 ± 0.03
	5,000	4	4.2 ± 0.2	0.3 ± 0.03	0.6 ± 0.04	1.3 ± 0.10
Female	0	4	4.2 ± 0.4	0.4 ± 0.03	0.6 ± 0.03	1.2 ± 0.06[ns]
	2,000	4	4.4 ± 0.2	0.4 ± 0.02	0.6 ± 0.04	1.1 ± 0.01
	5,000	4	4.1 ± 0.2	0.4 ± 0.04	0.6 ± 0.02	1.1 ± 0.07

[a]Values are expressed as means ± SEM; *ns* not significant

In addition to macroscopic evaluation, the major organs of the mice were also weighed. Organ weight is a basic measurement to diagnose whether the organ was exposed to the toxic substance or not. The liver, spleen, lung, and kidney are the major organs affected by metabolism caused by toxic substances (Dybing et al. 2002). Compared to the control group, the organ weights of liver, spleen, lung, and kidney from the mice administered T-Xyl indicated no significant changes (Tables 2 and 3).

3.4 Histopathological Examination

Because the liver is very important in the metabolism of toxic substances, we conducted microscopic examination of liver sections. No abnormal findings or lesions upon macroscopic examination were observed.

In this study, no significant differences in histopathology of liver tissues between the experimental groups and the control group were observed (Figs. 2 and 3)

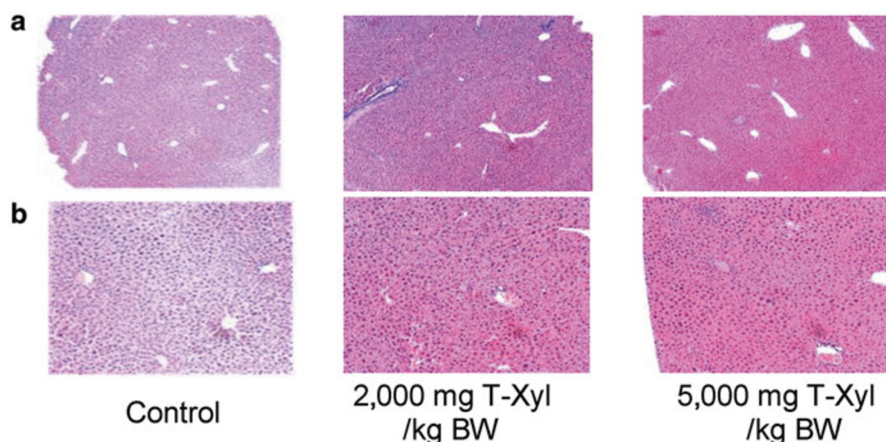

Fig. 2 Light microscopy of hepatocytes in male ICR mice. (**a**) ×100 magnification (**b**) ×200 magnification. Images are representative H&E-stained liver sections. No significant differences were found in liver histology between the different treatment groups

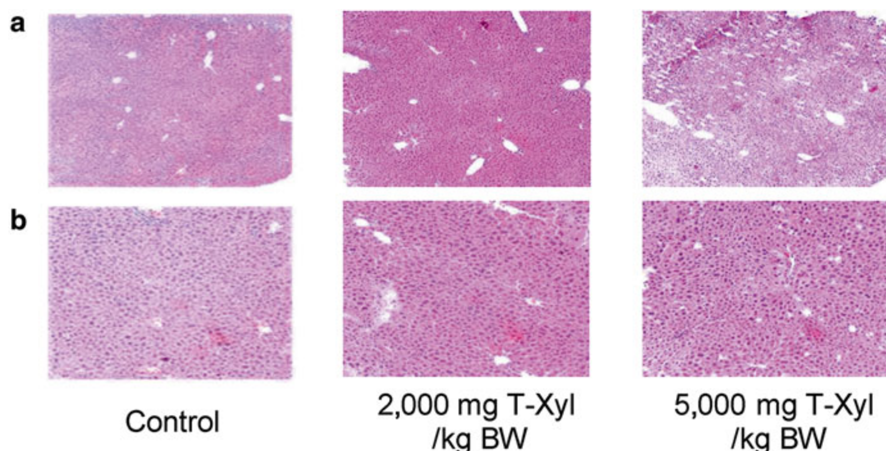

Fig. 3 Light micrography of hepatocytes in female ICR mice. (**a**) ×100 magnification (**b**) ×200 magnification. Images are representative H&E-stained liver sections. No significant differences were found in liver histology between the different treatment groups

4 Conclusion

In this study, we assessed the safety of T-Xyl in male and female ICR mice. Our results showed that the LD_{50} value of T-Xyl was >5,000 mg/kg BW and that an acute oral administration of T-Xyl was not toxic in male or female ICR mice. Therefore, oral administration of T-Xyl has the potential to be safe for clinical use, but further toxicity studies of repeated oral dosing are needed.

Acknowledgements We thank Dong-A Pharmaceutical Co. for the donation of taurine.

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Anti-obesity and Hypolipidemic Effects of N-(β-D-Xylopyranosyl)Taurine Sodium Salt Supplementation in Rats Fed a High-Fat Diet and β-Alanine

Jeong Soon You, Yun Ju Lee, So Young Kim, Sung Hoon Kim, and Kyung Ja Chang

Abbreviations

T-Xyl	*N*-(*β*-D-Xylopyranosyl)taurine sodium salt
BW	Body weight
N	Normal diet
HF	High-fat diet
HFT	High-fat diet + 4 mmol/kg/day taurine
HFA	High-fat diet + 3 % β-alanine
HFTX2	High-fat diet + 3 % β-alanine + 2 mmol/kg/day T-Xyl
HFTX4	High-fat diet + 3 % β-alanine + 4 mmol/kg/day T-Xyl
HFTX6	High-fat diet + 3 % β-alanine + 6 mmol/kg/day T-Xyl
E-fat	Epididymal fat
R-fat	Retroperitoneal fat
TG	Triglyceride
TC	Total cholesterol
HDLC	High density lipoprotein cholesterol
LDLC	Low density lipoprotein cholesterol
AI	Atherogenic index
H&E	Hematoxylin and eosin
SEM	Standard error of mean

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1 Introduction

Obesity has emerged as a social problem and its biological and socio-economic cost is increasing rapidly worldwide. Obesity is closely associated with many chronic diseases, including hypertension, cardiovascular disease, diabetes mellitus, atherosclerosis, and various cancers (Hall 2003; Achike et al. 2011) and with increased mortality (Rahmouni et al. 2005).

Taurine, a sulfur-containing amino acid, is formed from cysteine in human hepatocytes and is present at high concentration in all animal tissue (Sturman 1993). Taurine has been reported to possess biological activities (Schurr et al. 1987; Chang 2002; Foos and Wu 2002), including antioxidant, bile acid conjugation, recovery of neuronal function, calcium homeostasis, and antidiabetic effects. For this reason, the use of taurine-containing drinks, health functional food, and therapeutic agents is increasing. However, disadvantages associated with taurine, such as poor absorption and a rapid renal extraction rate, have also been reported (Cho et al. 2014). In an effort to overcome these disadvantages, numerous taurine derivatives have been developed and various functions have been reported. In particular, considering the absorption through the carbohydrate transporter, taurine-carbohydrate derivatives were developed and an anti-adipogenic effect of T-Xyl was verified *in vitro* (Cho et al. 2014).

Therefore, this study was conducted in order to evaluate the anti-obesity and hypolipidaemic effects of T-Xyl using *in vivo* experiments. For *in vivo* experiments, T-Xyl was administered orally to rats fed a high-fat diet; we measured body and adipose tissue weights and serum lipid levels. In addition, to ensure absorption through a transporter other than the taurine transporter, β-alanine, which competes with taurine and induces taurine depletion, was also administered with T-Xyl to rats.

2 Materials and Methods

2.1 Preparation of T-Xyl and Animals

N-(β-D-Xylopyranosyl)taurine sodium salt was a newly synthesized taurine-carbohydrate derivative, as described previously (Cho et al. 2014). The reaction of D-xylose with taurine was carried out and the chemical structure of T-Xyl was identified by nuclear magnetic resonance spectroscopy.

Four-week-old male Sprague-Dawley rats were purchased from Koatech (Pyeongtaek, Korea). All rats were kept in laboratory animal housing at Inha University and the procedures of mice care were performed with a constant 12 h light and dark cycle (09:00 a.m. to 09:00 p.m.), controlled temperature (22 ± 2 °C), and humidity (55 ± 5 %). All procedures of animal care were performed in accordance with the guidelines established by the Experimental Animal Ethics Committee of Inha University.

2.2 Experimental Design

Following 1 week of acclimatization with a commercial pellet diet, rats were randomly divided into seven groups (normal diet, N group; high-fat diet, HF group; high-fat diet + 4 mmol/kg/day taurine, HFT group; high-fat diet + 3 % β-alanine, HFA group; high-fat diet + 3 % β-alanine + 2, 4, 6 mmol/kg/day T-Xyl, HFTX2, 4, 6 groups) with eight rats per group and fed an experimental diet (Table 1) by AIN-93 (Reeves et al. 1993) and water *ad libitum*. Vitamin and mineral mixture were purchased from Feedlab (Guri, Korea).

Taurine and T-Xyl were administered orally to rats in the HFT and HFTXs groups and the same amount of distilled water was provided orally to rats in the N, HF, and HFA groups. β-alanine was supplemented by dissolving in tap water (3 % w/v). Food and water intake was measured twice per week. The feed efficiency ratio (FER) was calculated as weight gain in grams divided by dietary intake in grams and body weight was measured once per week.

2.3 Sampling and Chemical Analysis

After 6 weeks, the rats were fasted for 12 h before sacrifice. Blood that was collected from the heart and serum was obtained by centrifugation at 1,650 × *g* for 20 min. Epididymal fat (E-fat) and retroperitoneal fat (R-fat) were weighed. Some E-fat was removed from the rats for histological photography and serum was stored at −70 °C until application.

Analysis of serum triglyceride (TG) and total cholesterol (TC) level was performed using an automatic analyzer (BPC BioSed srl, Rome, Italy). High density

Table 1 Composition of experimental diet (g/100 g diet)

	Experimental diets	
Component	Normal diet (g)	High-fat diet (g)
Casein	200	200
Corn starch	529.486	105.036
Sucrose	100	232
Cellulose	50	50
Lard	0	175
Soybean oil	70	25
AIN-93 mineral mixture	35	35
AIN-93 vitamin mixture	10	10
DL-Methionine	3	3
Choline bitartrate	2.5	2.5
Tert-butyl hydroquinone	0.014	0.014
Total	1,000	837.55

lipoprotein cholesterol (HDLC) was obtained from whole serum using a high-density lipoprotein precipitation reagent (Asan Pharm Co., Gyeonggi, Korea) after precipitation of low density lipoprotein and very low density lipoprotein for 10 min at $800 \times g$, followed by analysis for HDLC using the same method employed for TC. Serum low density lipoprotein cholesterol (LDLC) value was calculated using the following formula (Friedewald et al. 1972):

$$LDLC = TC - (HDLC + TG / 5)$$

Assayed chemistry control (Bio-Rad Laboratories, Irvine, CA, USA) was used for calibration and all of the results were expressed as mg/dL serum.

Atherogenic Index ((TC-HDLC)/HDLC) is an important prognostic marker for future cardiovascular disease. A higher index indicates increased cardiovascular risk (Haglund et al. 1991).

2.4 Histological Photograph of Adipose Tissue

Histological photograph of epididymal adipose tissue was examined under light microscope (Axioskop 2, Zeiss, Jena, Germany) using the paraffin method. Fresh tissues were fixed immediately with 10 % buffered formalin, and paraffin-embedded sections were stained with hematoxylin and eosin (H&E).

2.5 Statistical Analysis

Data were expressed as the mean ± standard error of the mean (SEM) and one-way analysis of variance was used, followed by Duncan's multiple range tests at $p < 0.05$ for analysis to determine significant difference. All analyses were performed using the SPSS 20.0 program.

3 Results and Discussion

3.1 Effect of T-Xyl on Body Weight

To examine the anti-obesity effect of T-Xyl, we fed rats a high-fat diet for 6 weeks. Taurine depletion was reported to induce obesity (Tsuboyama-Kasaoka et al. 2006). At the end of the experiment, rats fed a high-fat diet or a high-fat diet with β-alanine had a significantly higher body weight than rats fed a normal diet. However, administration of taurine or T-Xyl resulted in suppression of high-fat diet induced body weight gain (Table 2). In particular, T-Xyl suppressed body weight more effectively than taurine.

Table 2 Effects of T-Xyl on body weight and body weight gain

Group	Initial body weight (g)	Final body weight (g)	Body weight gain (g/6 weeks)
N	188.3 ± 1.9^{ns}	348.6 ± 11.8^{a}	161.3 ± 10.3^{a}
HF	187.3 ± 6.1	402.7 ± 6.6^{bc}	210.6 ± 4.4^{c}
HFT	186.7 ± 6.5	379.4 ± 12.9^{abc}	192.7 ± 9.2^{bc}
HFA	191.6 ± 5.9	407.1 ± 17.8^{c}	213.2 ± 10.9^{c}
HFTX2	189.3 ± 6.3	369.7 ± 3.6^{ab}	180.4 ± 6.1^{ab}
HFTX4	192.4 ± 4.3	368.6 ± 10.4^{ab}	177.5 ± 6.2^{ab}
HFTX6	192.0 ± 4.3	366.5 ± 11.5^{ab}	174.6 ± 7.9^{ab}

Values are expressed as mean ± SEM. Values with different superscript indicators within the column are significantly different at $p < 0.05$ by Duncan's multiple range test; *ns* not significant
N normal diet; *HF* high-fat diet; *HFT* high-fat diet + 4 mmol/kg/day taurine; *HFA* high-fat diet + 3 % β-alanine; *HFTX2, 4, 6* high-fat diet + 3 % β-alanine + 2, 4, 6 mmol/kg/day T-Xyl

3.2 Effects of T-Xyl on Diet Intake, Water Intake, and FER

No significant difference in diet intake was observed between the N and HF groups; however, administration of 6 mmol/kg/day T-Xyl appeared to result in a decrease in food intake, which was increased in the HFA group (Table 3). In a previous report (Chung et al. 2003), administration of 1.5 or 2 % taurine to rats did not affect diet intake.

The FER of the HF group was significantly higher, compared to the N group, while those of HFTX4 and HFTX6 were significantly lower, compared to the HF group. In addition, administration of taurine, β-alanine, or 2 mmol/kg/day T-Xyl tended to decrease FER.

3.3 Effects of T-Xyl on Adipose Tissues

In previous studies, the E-fat weight of the HF group was higher compared to the N group, and a positive correlation was observed between BW and body fat (Tsuboyama-Kasaoka et al. 2006). In this study, E-fat and R-fat, a large portion of body fat, was weighed and relative weight was calculated (Fig. 1). The relative weights of E-fat of the HF and HFA groups were higher, compared to the N group, and those of the HFT and HFTXs groups tended to be lower, but not significantly lower, compared to the HF group. No significant difference in the relative weight of R-fat was observed among all the groups.

In microscopic examination (Fig. 2), epididymal adipocytes showed an irregular and large histological appearance in the HF and HFA groups, compared to the N group. However, this morphological change was not observed in the HFT and HFTXs groups. Therefore, taurine and T-Xyl may inhibit the increase of adipocyte size induced in rats fed a high-fat diet.

Table 3 Effects of T-Xyl on diet intake, water intake, and FER

Group	Food intake (g/day)	Food intake (Cal /day)	Water intake (g/day)	FER (%)
N	20.8 ± 1.3^{ab}	83.1 ± 5.3^{a}	25.4 ± 1.3^{a}	21.4 ± 0.8^{a}
HF	18.2 ± 0.7^{ab}	86.8 ± 3.4^{ab}	30.3 ± 3.4^{ab}	27.0 ± 0.7^{b}
HFT	21.5 ± 1.7^{ab}	102.6 ± 7.9^{b}	33.5 ± 1.9^{b}	24.6 ± 1.0^{ab}
HFA	21.9 ± 1.5^{b}	104.6 ± 7.4^{b}	31.2 ± 2.6^{ab}	24.4 ± 1.9^{ab}
HFTX2	18.5 ± 0.9^{ab}	88.2 ± 4.3^{ab}	30.6 ± 1.9^{ab}	23.6 ± 0.9^{ab}
HFTX4	18.7 ± 0.9^{ab}	89.5 ± 4.3^{ab}	35.8 ± 2.4^{b}	22.8 ± 0.5^{a}
HFTX6	17.6 ± 1.3^{a}	84.1 ± 6.1^{a}	32.7 ± 2.0^{ab}	21.7 ± 1.2^{a}

Values are expressed as mean ± SEM. Values with different superscript indicators within the column are significantly different at $p < 0.05$ by Duncan's multiple range test
N normal diet; *HF* high-fat diet; *HFT* high-fat diet + 4 mmol/kg/day taurine; *HFA* high-fat diet + 3 % β-alanine; *HFTX2, 4, 6* high-fat diet + 3 % β-alanine + 2, 4, 6 mmol/kg/day T-Xyl; *FER* feed efficiency ratio

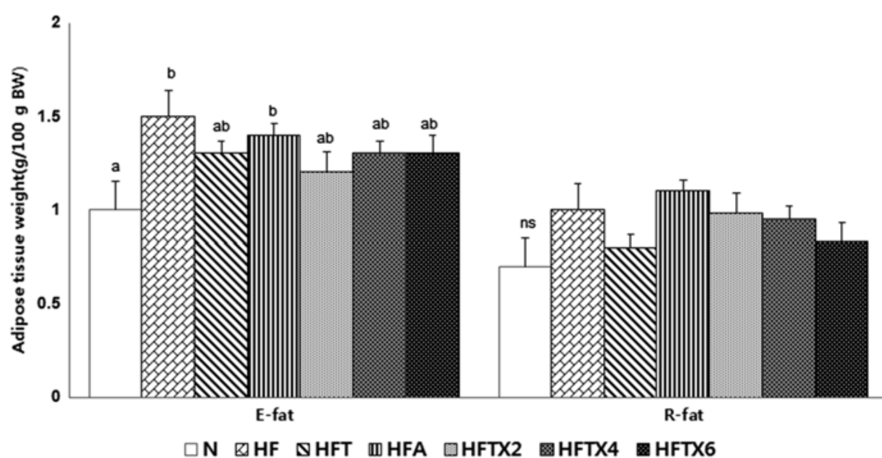

Fig. 1 Effects of T-Xyl on weights of adipose tissue. Values are expressed as mean ± SEM, *N* normal diet; *HF* high-fat diet; *HFT* high-fat diet + 4 mmol/kg/day taurine; *HFA* high-fat diet + 3 % β-alanine; *HFTX2, 4, 6* high-fat diet + 3 % β-alanine + 2, 4, 6 mmol/kg/day T-Xyl; *E-fat* epididymal fat; *R-fat* retroperitoneal fat. Values with different superscript indicators are significantly different at $p < 0.05$ by Duncan's multiple range test; *ns* not significant

It is known that the number of adipocytes formed cannot be decreased by diet, however, the size of adipocytes can adjust (Kang et al. 2004). When adipocyte size was measured using the ImageJ program (Fig. 3), those of the HF group were significantly larger than those of the N group; however, administration of taurine and T-Xyl resulted in a decrease in adipocyte size. In particular, administration of T-Xyl appeared to result in a dose-dependent decrease in size. Therefore, administration of both T-Xyl and taurine in rats fed a high-fat diet resulted in a decrease in weight of E-fat and in adipocyte size.

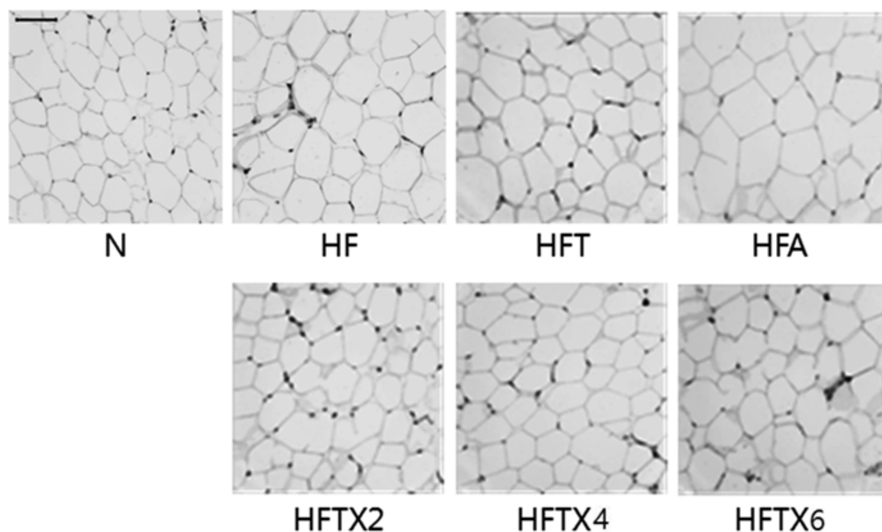

Fig. 2 Effect of T-Xyl on histological photograph of epididymal fat. *N* normal diet; *HF* high-fat diet; *HFT* high-fat diet+4 mmol/kg/day taurine; *HFA* high-fat diet+3 % β-alanine; *HFTX2, 4, 6* high-fat diet+3 % β-alanine+2, 4, 6 mmol/kg/day T-Xyl. Representative pictures of H&E-stained sections of epididymal adipocytes in N, HFT, and HFTXs; adipocyte size is smaller in N, HFT and HFTXs than in HF rats (400× magnification), Scale bar: 100 μm

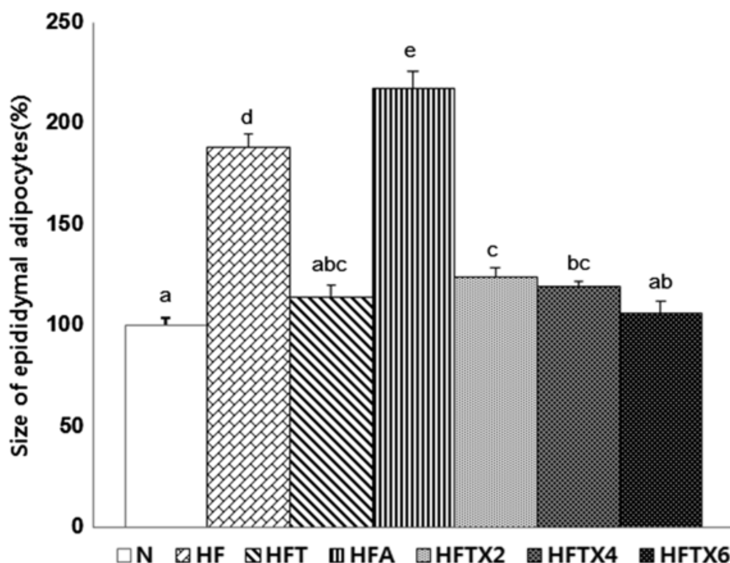

Fig. 3 Effect of T-Xyl intake on relative size of epididymal adipocyte. Values are expressed as mean±SEM, *N* normal diet; *HF* high-fat diet; *HFT* high-fat diet+4 mmol/kg/day taurine; *HFA* high-fat diet+3 % β-alanine; *HFTX2, 4, 6* high-fat diet+3 % β-alanine+2, 4, 6 mmol/kg/day T-Xyl. Values with different superscript indicators are significantly different at $p<0.05$ by Duncan's multiple range test

3.4 Effects of T-Xyl on Serum Lipid Levels

Results for serum lipid levels are shown in Table 4. The HA group showed a significant increase in serum TC level compared to the N group; however, no significant differences in TG and LDLC levels were observed among the groups. Higher serum HDLC levels are known to prevent cardiovascular disease. Administration of taurine or T-Xyl tended to increase while 6 mmol/kg/day T-Xyl significantly decreased serum HDLC levels.

Some studies have reported that serum TC and LDLC levels were increased in taurine depletion diabetic rats and administration of taurine in rats fed a high-fat diet resulted in a decrease in serum LDLC and an increase in serum HDLC (Murakami et al. 1998). In addition, administration of 3 g taurine to obese college students for 7 weeks resulted in a decrease in serum TG levels (Zhang et al. 2004).

The values for AI (Table 5), an important prognostic marker for future cardiovascular disease, are shown in Table 5. AI tends to be higher in the HF group and is significantly increased in the HFA group; however, administration of T-Xyl appeared to decrease AI.

In this study, taurine depletion increased serum TC level and AI index while administration of both T-Xyl and taurine improved serum lipid levels in rats fed a high-fat diet. In particular, administration of T-Xyl was more effective than taurine in rats fed a high-fat diet and β-alanine.

Table 4 Effects of T-Xyl on serum lipid levels

Group	TG (mg/dL)	TC (mg/dL)	LDLC (mg/dL)	HDLC (mg/dL)
N	53.1 ± 8.1^{ns}	104.0 ± 4.5^{a}	50.7 ± 5.6^{ns}	42.7 ± 2.9^{ab}
HF	63.3 ± 16.0	119.8 ± 5.5^{ab}	60.9 ± 3.6	38.8 ± 1.0^{a}
HFT	54.4 ± 10.7	110.2 ± 6.2^{ab}	54.1 ± 5.8	45.3 ± 3.4^{ab}
HFA	68.0 ± 15.9	123.7 ± 8.1^{b}	63.7 ± 7.7	41.6 ± 3.2^{ab}
HFTX2	62.8 ± 4.0	111.6 ± 4.5^{ab}	56.6 ± 4.6	45.5 ± 1.7^{ab}
HFTX4	48.0 ± 8.1	105.3 ± 4.9^{ab}	54.9 ± 4.4	45.8 ± 1.0^{ab}
HFTX6	48.1 ± 4.8	110.7 ± 2.9^{ab}	52.3 ± 3.5	48.0 ± 2.7^{b}

Values are expressed as mean ± SEM. Values with different superscript indicators within the column are significantly different at $p < 0.05$ by Duncan's multiple range test; *ns* not significant
N normal diet; *HF* high-fat diet; *HFT* high-fat diet + 4 mmol/kg/day taurine; *HFA* high-fat diet + 3 % β-alanine; *HFTX2, 4, 6* high-fat diet + 3 % β-alanine + 2, 4, 6 mmol/kg/day T-Xyl; *TG* triglyceride; *TC* total cholesterol; *HDLC* high density lipoprotein cholesterol; *LDLC* low density lipoprotein cholesterol

Table 5 Effects of T-Xyl on AI

Group	AI
N	1.5 ± 0.2^{ab}
HF	2.0 ± 0.2^{bc}
HFT	1.5 ± 0.2^{ab}
HFA	2.1 ± 0.1^{c}
HFTX2	1.6 ± 0.2^{ab}
HFTX4	1.4 ± 0.2^{a}
HFTX6	1.4 ± 0.2^{a}

Values are expressed as mean ± SEM. Values with different superscript indicators are significantly different at $p < 0.05$ by Duncan's multiple range test
N normal diet; *HF* high-fat diet; *HFT* high-fat diet + 4 mmol/kg/day taurine; *HFA* high-fat diet + 3 % β-alanine; *HFTX2, 4, 6* high-fat diet + 3 % β-alanine + 2, 4, 6 mmol/kg/day T-Xyl; *AI* atherogenic index

4 Conclusion

Based on our study, these results suggest that administration of both T-Xyl and taurine show anti-obesity and hypolipidemic effects in rats fed a high-fat diet and β-alanine. We also verified that T-Xyl can be taken up via a transporter other than the taurine transporter; further study is needed in order to clarify the mechanism of T-Xyl's action.

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N-(D-Ribopyranosyl)Taurine Sodium Salt Has Anti-obesity Effect in Diet-Induced Obesity and Taurine Deficiency Rat Model

So Young Kim, Yun Ju Lee, Jeong Soon You, Sung Hoon Kim, and Kyung Ja Chang

Abbreviations

T-Rib	*N*-(D-Ribopyranosyl)taurine sodium salt
N	Normal diet
HF	High-fat diet
HFT	High-fat diet + taurine (4 mmol/kg/day)
HFA	High-fat diet + β-alanine
HFR2	High-fat diet + β-alanine + T-Rib (2 mmol/kg/day)
HFR4	High-fat diet + β-alanine + T-Rib (4 mmol/kg/day)
HFR6	High-fat diet + β-alanine + T-Rib (6 mmol/kg/day)
FER	Food efficiency ratio
BW	Body weight
E-fat	Epididymal fat
R-fat	Retroperitoneal fat
H&E	Hematoxylin and eosin
TC	Total cholesterol
TG	Triglyceride
HDL-C	High density lipoprotein cholesterol
LDL-C	Low density lipoprotein cholesterol
SE	Standard error

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1 Introduction

Obesity is defined as the accumulation of excess body fat resulting from high-fat and high sucrose diets (Grundy 1998). Obesity not only causes a change in body shape but also dyslipidemia and cardiovascular disease (Kopelman 2000; Furukawa et al. 2004). In addition, increasing child and adolescent obesity is a serious health problem and a risk factor for type 2 diabetes (Sinha et al. 2002) and cardiovascular disease (Boyd et al. 2005). Therefore, prevention of obesity in children and adolescents is important.

Taurine, an abundant amino acid in mammalian tissue, has various physiological functions, including antioxidant, development of brain, improvement of dyslipidemia, anti-obesity, etc. (Huxtable 1992; Chang et al. 2010; Chen et al. 2012).

Generally, in healthy humans, the average amount of taurine synthesis is 50–125 mg daily (Jacobsen and Smith 1968) and animal foods, such as meat and seafood, are the main sources of taurine. However, those who do not eat these foods regularly, particularly vegetarians, may be at risk for taurine deficiency (Laidlaw et al. 1988). Taurine deficiency may be caused by vitamin B_6 deficiency, parenteral nutrition, enzymatic immaturity, and disorders such as hepatic dysfunction and chronic renal failure (Shin and Linkswiler 1974; Geggel et al. 1985; Bergstrom et al. 1989; Martensson et al. 1992; Miller et al. 1995; Vina et al. 1995). Therefore, in a specific case, adequate intake of foods containing taurine or taurine supplementations is necessary.

N-(D-Ribopyranosyl)taurine sodium salt (Fig. 1), a synthetic product derived from taurine, consists of taurine and ribose. Therefore, it is believed that T-Rib is taken up into the transport of mono-saccharide located in tissues. It was also reported that T-Rib inhibited *in vitro* adipocyte differentiation and accumulation in human pre-adipocytes (Cho et al. 2014).

The purpose of this study was to investigate the anti-obesity effects of taurine supplementation in diet-induced obesity and in a taurine deficiency rat model.

2 Methods

2.1 Animals and Diet

Four-week old male Sprague-Dawley rats were obtained from Koatech (Pyeongtaek, Korea) and were kept in laboratory animal housing at Inha University with a constant 12 h light and dark cycle (AM 08:00 ~ PM 08:00), controlled temperature

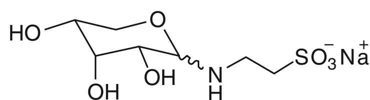

Fig. 1 Structure of T-Rib

Table 1 Composition of experimental diets (g/100 g diet)

	Experimental diets	
Ingredients	Normal diet (g)	High-fat diet (g)
Casein	200	200
Corn starch	529.486	105.036
Sucrose	100	232
Cellulose	50	50
Lard	0	175
Soybean oil	70	25
AIN-93 mineral mixture	35	35
AIN-93 vitamin mixture	10	10
DL-Methionine	3	3
Choline bitartrate	2.5	2.5
Tert-butyl hydroquinone	0.014	0.014
Total	1,000	837.55

(22 ± 2 °C) and humidity (60 ± 5 %). Following 1 week of acclimatization with a chow diet, rats were randomly divided into seven groups for a period of 6 weeks {normal diet, N group; high-fat diet, HF group; high-fat diet + taurine (4 mmol/kg/day), HFT group; high-fat diet + 3 % β-alanine, HFA group; high-fat diet + 3 % β-alanine + T-Rib (2 mmol/kg/day), HFR2 group; high-fat diet + 3 % β-alanine + T-Rib (4 mmol/kg/day), HFR4 group; high-fat diet + 3 % β-alanine + T-Rib (6 mmol/kg/day), HFR6 group}. Taurine and T-Rib were orally administered to the HFT, HFR2, HFR4, and HFR6 groups and the same amount of distilled water was orally administered to the other groups. β-alanine was supplemented by dissolving in tap water (3 % w/v) for induction of taurine deficiency.

Food and water were provided *ad libitum*. The composition of the experimental diet was based on AIN 93G (Reeves et al. 1993), as shown in Table 1. AIN-93 mineral and vitamin mixture were obtained from Feedlab (Guri, Korea). Food and water intake was measured per week and the feed efficiency ratio (FER) was calculated as weight gain in grams divided by dietary intake in grams. Body weight (BW) was measured once per week.

2.2 Sampling and Chemical Analysis

The animals were sacrificed after 6 weeks of feeding the experimental diets. Blood was collected from the heart and serum was separated by centrifuging at 3,000 rpm for 20 min. The sera were immediately frozen in liquid nitrogen, and then stored at −70 °C before analysis. The weights of the liver, epididymal fat (E-fat), and retroperitoneal fat (R-fat) were measured.

Epididymal adipose tissue stained with hematoxylin and eosin (H&E) was examined by light microscopy (Axioskop 2, ZEISS, Jena, Germany). All images were

captured using a digital capture system (spot flex fx 1520, SPOT Imaging Solution, Michigan, USA). The size of cells was calculated using the Image J program (National Institutes of Health, Maryland, USA).

Concentrations of serum triglyceride (TG), total cholesterol (TC), and high density lipoprotein cholesterol (HDL-C) were analyzed using an automatic analyzer (BPC BioSed srl, Italy). Low density lipoprotein cholesterol (LDL-C) was calculated according to Friedewald (1972). Standard serum (Asan Pharmaceutical, Korea) was used for calibration before analysis of each parameter. All of the results were expressed as mg/dl serum.

2.3 Statistical Analysis

All analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA) statistical package. Each value was expressed as the mean ± standard error (SE). Data were analyzed for significant difference by one-way analysis of variation followed by Duncan's multiple range tests at a $p < 0.05$.

3 Results and Discussion

3.1 Body Weight Gain, Food Intake, and FER

Body weight gain, food intake, and FER are shown in Table 2. After 6 weeks, body weight gain was significantly higher in the HF group compared to the N group, due to the high-fat diet containing 45 % energy from fat. In rats administered T-Rib at

Table 2 Body weight gain, food intake, FER

Group	Body weight gain (g/6 weeks)	Food intake (g/day)	FER (%)
N	166.1 ± 11.0^{a}	18.0 ± 1.2^{ns}	20.2 ± 1.1^{a}
HF	210.6 ± 4.4^{c}	17.9 ± 0.4	25.5 ± 1.1^{c}
HFT	197.8 ± 7.2^{bc}	18.3 ± 0.4	25.8 ± 0.6^{c}
HFA	211.3 ± 9.2^{c}	21.8 ± 1.1	24.2 ± 1.6^{bc}
HFR2	180.0 ± 8.9^{ab}	20.3 ± 1.2	21.3 ± 1.2^{ab}
HFR4	176.9 ± 3.7^{ab}	18.8 ± 0.5	22.8 ± 0.7^{abc}
HFR6	167.2 ± 5.4^{a}	19.8 ± 1.3	19.4 ± 1.8^{a}

Values are expressed as mean ± SE; Values with different superscripts within the column are significantly different at $p < 0.05$, as determined by Duncan's multiple range test; ns is not significant

FER (Food efficiency ratio) = total body weight gain × 100/total food intake

N normal diet group, *HF* high-fat diet group, *HFT* high-fat diet + 4 mmol/kg/day taurine group, *HFA* high-fat diet + 3 % β-alanine group, *HFR2* high-fat diet + 3 % β-alanine + 2 mmol/kg/day T-Rib group, *HFR4* high-fat diet + 3 % β-alanine + 4 mmol/kg/day T-Rib group, *HFR6* high-fat diet + 3 % β-alanine + 6 mmol/kg/day T-Rib group

Table 3 Liver weight and adipose tissue weight

Group	Relative liver (g/100 g BW)	Relative epididymal fat (g/100 g BW)	Relative retroperitoneal fat (g/100 g BW)
N	2.8 ± 0.2^{ns}	1.0 ± 0.1^{a}	0.7 ± 0.1^{a}
HF	3.1 ± 0.1	1.5 ± 0.1^{d}	1.2 ± 0.2^{b}
HFT	2.9 ± 0.2	1.3 ± 0.1^{bcd}	0.8 ± 0.2^{ab}
HFA	3.2 ± 0.2	1.4 ± 0.1^{d}	1.2 ± 0.1^{b}
HFR2	2.9 ± 0.2	1.3 ± 0.1^{cd}	1.0 ± 0.0^{ab}
HFR4	3.0 ± 0.1	1.1 ± 0.1^{abc}	0.9 ± 0.1^{ab}
HFR6	3.0 ± 0.1	1.0 ± 0.1^{ab}	0.8 ± 0.1^{a}

Values are expressed as mean ± SE; Values with different superscripts within the column are significantly different at $p < 0.05$, as determined by Duncan's multiple range test; ns is not significant
N normal diet group, *HF* high-fat diet group, *HFT* high-fat diet + 4 mmol/kg/day taurine group, *HFA* high-fat diet + 3 % β-alanine group, *HFR2* high-fat diet + 3 % β-alanine + 2 mmol/kg/day T-Rib group, *HFR4* high-fat diet + 3 % β-alanine + 4 mmol/kg/day T-Rib group, *HFR6* high-fat diet + 3 % β-alanine + 6 mmol/kg/day T-Rib group

doses of 4 and 6 mmol/kg/day, the body weight gain was significantly lower compared to the HFA group. No significant differences in daily food intake were observed among the groups during the experimental period. However, FER in the HFR6 group was significantly lower compared to the HF group, at levels similar to those of the N group. These results suggest that T-Rib could inhibit body weight gain in a high-fat diet.

3.2 Liver Weight and Adipose Tissues Weights

The liver and adipose tissues weights are shown in Table 3. No significant differences in the relative liver weight were observed among the groups. The adipose tissue weights (E-fat and R-fat) were significantly higher in the HF group and the HFA group compared to the N group. In particular, β-alanine supplementation for inducing a taurine deficiency did not affect adipose tissue weights of rats fed a high-fat diet.

However, in rats administered in the T-Rib group, particularly in the HFR6 group, relative E-fat and R-fat weights were significantly lower compared to the HF and HFA groups. These data indicated that T-Rib supplementation decreased not only the body weight gain but also adipose tissue weights.

Adipose tissue is a storage site for excess energy and secretes various adipokines (Hajer et al. 2008). One of the adipokines, leptin, with pro-inflammatory properties is over-produced (Considine et al. 1996) and the other adipokine, adiponectin, with anti-inflammatory activity is decreased in obesity (Arita et al. 1999; Yamauchi et al. 2001). These states may promote obesity-linked metabolic syndromes. Therefore, T-Rib supplementation can prevent obesity and diseases related to obesity, such as dyslipidemia, hyperinsulinemia, and atherosclerosis.

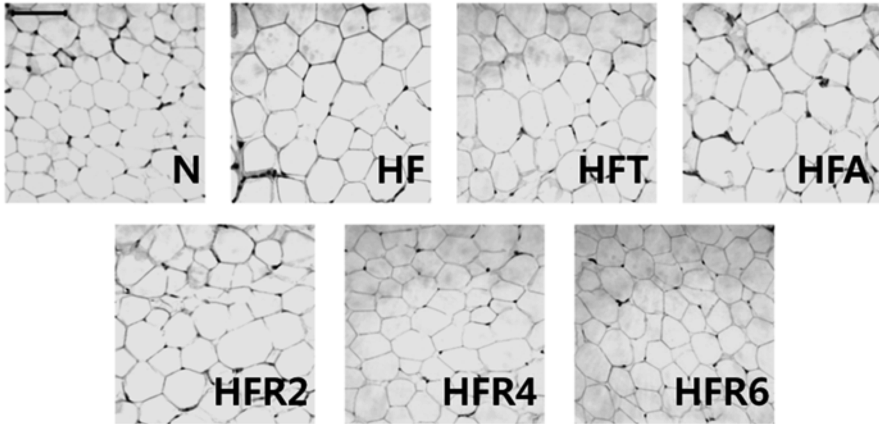

Fig. 2 Light micrographs of epididymal adipocytes in rats fed a high-fat diet. Representative images of hematoxylin and eosin stained sections of epididymal adipocytes from experimental groups. Scale bar = 100 μm. *N* normal diet group, *HF* high-fat diet group, *HFT* high-fat diet + 4 mmol/kg/day taurine group, *HFA* high-fat diet + 3 % β-alanine group, *HFR2* high-fat diet + 3 % β-alanine + 2 mmol/kg/day T-Rib group, *HFR4* high-fat diet + 3 % β-alanine + 4 mmol/kg/day T-Rib group, *HFR6* high-fat diet + 3 % β-alanine + 6 mmol/kg/day T-Rib group

3.3 Adipose Tissue Histopathology

Light micrographs of epididymal adipocytes are shown in Fig. 2. Histological images of epididymal adipocytes showed differences in morphology among the experimental groups. Adipocytes in the HF and HFA groups had irregular shapes and varied in size, whereas shapes of adipocytes in the HFA4 and HFA6 groups were uniform and small, such as the N group.

The relative size of the adipocytes of the HF group was significantly increased compared to the N group (Fig. 3). However, relative size of adipocytes of the HFR6 group was as small as that of the N group. In addition, relative size of adipocytes was significantly lower in the HFR4 group compared to the HFT group. That is, T-Rib supplementation was more effective in reducing adipocyte size than taurine treatment at the same dose.

3.4 Serum Lipid Profiles

Serum lipid profiles are shown in Table 4. No significant differences in the concentration of serum TG and LDL-C were observed among the groups. The concentration of TC was significantly increased in the HF group compared to the N group. Beneficial effects of taurine on serum lipids have been noted in rats fed a high-fat diet (Murakami et al. 1998; Du et al. 2010; Nardelli et al. 2011). We have also shown that taurine supplementation improved the concentration of serum TC and HDL-C.

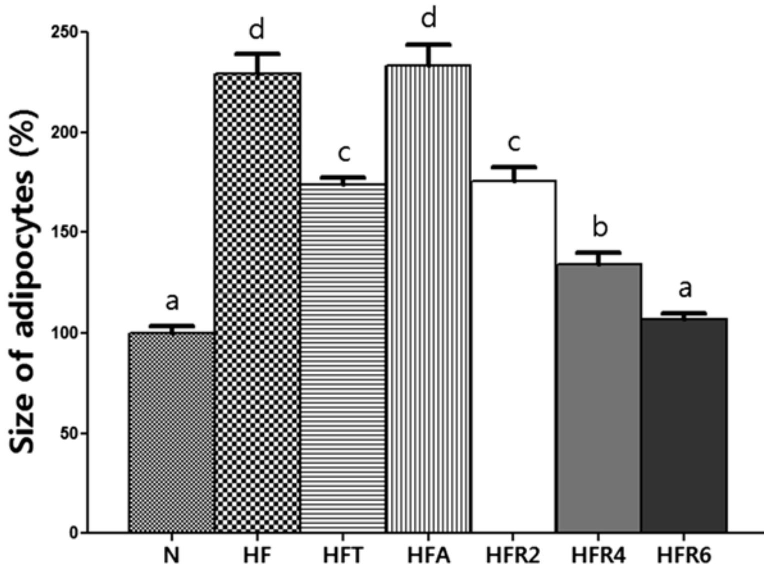

Fig. 3 Relative sizes of adipocytes. *N* normal diet group, *HF* high-fat diet group, *HFT* high-fat diet+4 mmol/kg/day taurine group, *HFA* high-fat diet+3 % β-alanine group, *HFR2* high-fat diet+3 % β-alanine+2 mmol/kg/day T-Rib group, *HFR4* high-fat diet+3 % β-alanine+4 mmol/kg/day T-Rib group, *HFR6* high-fat diet+3 % β-alanine+6 mmol/kg/day T-Rib group. Values are expressed as mean±SE; Values with different superscripts are significantly different at $p<0.05$, as determined by Duncan's multiple range test; ns is not significant

Table 4 Serum lipid profiles

Group	TG (mg/dL)	TC (mg/dL)	HDL-C (mg/dL)	LDL-C (mg/dL)
N	51.4 ± 4.6^{ns}	199.3 ± 2.2^{a}	43.8 ± 1.9^{ab}	49.6 ± 5.4^{ns}
HF	63.4±9.6	115.9 ± 3.2^{bc}	38.4 ± 0.9^{a}	58.4±4.0
HFT	58.3±6.3	105.0 ± 3.2^{ab}	46.5 ± 2.4^{b}	49.6±5.0
HFA	59.3±6.6	122.1 ± 5.0^{c}	38.5 ± 1.9^{a}	62.8±5.6
HFR2	52.1±5.1	111.8 ± 1.9^{b}	42.0 ± 1.4^{ab}	58.3±3.4
HFR4	55.2±5.7	110.6 ± 2.7^{b}	42.8 ± 1.6^{ab}	54.6±3.3
HFR6	56.6±5.5	106.8 ± 4.6^{ab}	44.7 ± 1.9^{b}	54.1±5.3

Values are expressed as mean±SE; Values with different superscripts within the column are significantly different at $p<0.05$, as determined by Duncan's multiple range test; ns is not significant
N normal diet group, *HF* high-fat diet group, *HFT* high-fat diet+4 mmol/kg/day taurine group, *HFA* high-fat diet+3 % β-alanine group, *HFR2* high-fat diet+3 % β-alanine+2 mmol/kg/day T-Rib group, *HFR4* high-fat diet+3 % β-alanine+4 mmol/kg/day T-Rib group, *HFR6* high-fat diet+3 % β-alanine+6 mmol/kg/day T-Rib group, *TG* triglyceride, *TC* total cholesterol, *HDL-C* high density lipoprotein cholesterol, *LDL-C* low density lipoprotein cholesterol

β-alanine is known to inhibit taurine reuptake into proximal tubular cells (Goldman and Scriver 1967; Dantzler and Silbernagl 1976). We used β-alanine to induce depletion of taurine. As a result, the concentration of serum TC was significantly increased and the concentration of HDL-C was significantly decreased in the HFA group compared to other groups. However, administration of T-Rib significantly lowered the concentration of serum TC. In addition, it significantly increased the concentration of serum HDL-C. These results suggest that T-Rib may be absorbed through another transporter instead of the taurine transporter. T-Rib supplementation may improve the concentration of serum TC and HDL-C depending on treatment dosage.

4 Conclusion

These results suggest that T-Rib supplementation has beneficial effects on body weight, adipose tissue weights, and serum TC and HDL-C concentration. Therefore, both T-Rib and taurine supplementation may have anti-obesity effects and T-Rib may be more effective than taurine in diet-induced obesity and in a taurine deficiency rat model.

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