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TAURINE 6

Edited by
Simo S. Oja
and
Pirjo Saransaari

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PREFACE

The 15th Taurine Meeting, "Taurine Today," was held June 12–15, 2005, in Tampere, Finland. Tampere is the third largest city (200,000 inhabitants) in Finland and the largest inland city in Scandinavia, located 109 miles north of Helsinki, capital of Finland. The meeting venue was the Scandic Hotel Rosendahl on the shores of the Lake Pyhäjärvi, located about two miles from the city center on a hillside of the highest sand ridge in the world. This narrow ridge was formed by the Ice Age between two large lakes, embracing the present-day city of Tampere.

Approximately 80 individuals attended this meeting from twenty-one countries and four continents. The present meeting continued the prestigious series of taurine meetings, regularly gathering together most of the prominent investigators in the field. The scientific program consisted of thirty-three platform and forty-seven poster presentations. This volume is based on these presentations given at the meeting. The topics range from the presence and metabolism of taurine in microorganisms to the applicability of taurine derivatives in clinical medicine. Characterization of the significance of taurine in nutrition and of the putative functions in the organisms was also emphasized, among several other aspects of taurine.

The Finnish Physiological Society and Tampere University Brain Research Center were the official organizations sponsoring the meeting. The Program Committee, Professors Junichi Azuma, Laura Della Corte, J. Barry Lombardini, Stephen W. Schaffer, Simo S. Oja, and Pirjo Saransaari, cooperated in composing the meeting program. The heaviest burden of all practical matters was on the shoulders of the Organizing Committee, including Prof. Simo S. Oja, Prof. Pirjo Saransaari, Assoc. Prof. Kirsi-Marja Marnela, Assoc. Prof. Vince Varga, Dr. Satoshi Abe, Dr. Sirpa Rainesalo, and Ms. Svetlana Molchanova, MSci.

The organizers of the meeting are indebted to the Academy of Finland; Tampere University Research Fund; Taisho Pharmaceutical Company, Ltd., Japan; Red Bull GmBh, Austria; Korean Taurine Society; Dong-A Pharmaceutical Company, Korea; and the organizer of the previous taurine meeting, Prof. Stephen Schaffer, for their generous financial support. The organizers likewise thank Ms. Svetlana Molchanova, MSci; Mr. Pasi Puumala, BMed; and Mr. Róbert Dohovics, MSci, for their skilful technical help with computers, Power Point shows, and microphones during the meeting. The excellent professional competence of the staff of the TAVI Congress Bureau, in particular that of, the managing director, Ms. Anja Hakkarainen, and the kind cooperation of the personnel of the Scandic Hotel Rosendahl, were greatly appreciated. Ms. Sari Luokkala gave us expert advice and technical help in editing the manuscripts.

Finally, the organizers wish to thank all the participants of the meeting and the authors of the papers in this volume. Their invaluable contributions made everything worth our efforts. The presentations at the meeting and the articles in this volume shed bright light on the various aspects of the significance and role of taurine, although many questions still remain unanswered. This calls for the next taurine meeting to be organized in about two years' time.

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CONTENTS

Part 1. Taurine Metabolism and Taurine Transporter

Part 2. Metabolic Effects of Taurine

Part 3. Effects of Taurine Supplementation

Part 4. Taurine in Heart and Muscles

Part 5. Taurine in Brain and Retina

Part 6. Taurine Derivatives

Part 1.
Taurine Metabolism and Taurine Transporter

METABOLISM OF TAURINE IN MICROORGANISMS

A Primer in Molecular Biodiversity?

Alasdair M. Cook and Karin Denger*

1. INTRODUCTION

To those studying the roles of taurine in mammals, one starting point might be Huxtable's review (Huxtable, 1992), in which about one page was devoted to a list of known and putative functions of the compound. Subsequent taurine meetings have added much to this picture. The review also supplies information for microbiologists; in the meantime, much has been learned about the roles of taurine in microbial metabolism, and I see it as my brief to introduce the facts of the matter, and to delineate fact and hypothesis.

First of all, I would like to remind you that we are, from the point of view of weight, mainly eukaryotic. However, from the point of view of numbers, we are largely prokaryotic. To be here, we should be able to perform considerable academic feats involving e.g. nerve cells and taurine, but metabolically, we are dunces: we can excrete it or we can conjugate it and excrete it. It is prokaryotes, apparently the bacteria, which show brilliance in manipulating taurine. So be warned that we are about to jump from one biogeochemical cycle to the next. Please fasten your seatbelts!

One of the first microbiologists to mention taurine was den Dooren de Jong in 1926 (den Dooren de Jong, 1926); he seems to have tested the compound as a source of nitrogen for growth, with success: we will return to the nitrogen cycle later. A key player was Kondo, who, with Shimamoto and Berk, set the scene for much of the present work in the carbon cycle by discovering sulfoacetaldehyde as a key intermediate (cited in Cook and Denger, 2002). Another key player is Kertesz (2000), who established the oxygenolytic desulfonation of taurine in the sulfur cycle. Mammals are used to aerobic conditions, but about half the biosphere (by weight) is anoxic, and many taurine utilizers grow under strictly anoxic conditions (Lie *et al*., 1998; Cook and Denger, 2002): be prepared for novel respirations and fermentations!

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We are used to taurine being the major organic solute in mammals (Huxtable, 1992) but think of the consequences for bacteria. When I drive you to tears, you are excreting taurine to feed bacteria. When I drive you to the toilet, your fluid and solid contributions to the sewage plant contain taurine and conjugated taurine. When I drive you to appendicitis, the taurine could well be utilized by *Bilophila wadsworthia* from your gut canal. When you try to wash me out of your hair, you are using taurine derivatives to generate the mild foam. When you get caught in a spider's web, one glue-auxiliary is taurine. And if you throw yourself into the ocean to escape, you will find taurine as an osmolyte in deep-sea creatures. At least one antibiotic, bulgecin A, is a taurine conjugate. Bacteria have many potential sources of taurine, but presumably usually at low concentrations.

2. TAURINE TRANSPORT INTO THE BACTERIAL CELL

Sulfonation has been described as Nature's way of keeping a compound on one side of a biological membrane (Graham *et al.*, 2002): the permanent negative charge in the physiological pH-range prevents passive diffusion across biological membranes. I gather that mammals have a relatively simple transporter, TauT [TC 2.A.22.3.3], to move taurine across membranes. Characterized enzymes of taurine biotransformation in bacteria are usually soluble, intracellular enzymes, so transport into the cell is essential for bacteria to be able to utilize the compound.

Bacteria seem to have complex transporters. The best-understood transport system for taurine is associated with the assimilation of taurine sulfur in *Escherichia coli*. This is a 3-component ATP-binding cassette transporter, i.e. an ABC transporter termed TauABC [TC 3.A.1.17.1] (Eichhorn *et al.*, 2000). The authors used mutation analysis and complementation studies to confirm the function of all three components. TauA is a periplasmic binding protein, TauC is the permease and TauB is the ATPase. This system, certainly as homologous genes and occasionally with further experimental support, is widespread in bacteria which utilize taurine sulfur for growth (Kertesz, 2001; Masepohl *et al.*, 2001).

We suspect that ABC transporters are also involved in the utilization of taurine as a carbon source. Currently the evidence is purely that of genes homologous to *tauABC* in e.g. *Sinorhizobium meliloti* and *Paracoccus pantotrophus*, and clustered with other genes known or believed to be involved with taurine dissimilation (Brüggemann *et al.*, 2004).

A different multi-component system is also believed to be involved in taurine transport in e.g. *Rhodobacter sphaeroides* and *Paracoccus denitrificans*. This is a tripartite ATP-independent (TRAP) system [TC 2.A.56.4.1] whose genes, *tauKLM*, are clustered with other genes known or believed to be involved with taurine dissimilation (Brüggemann *et al.*, 2004). There is, as yet, no experimental evidence for function, simply a sequence similarity to other TRAP transporters.

3. TWO ROUTES TO GENERATE SULFOACETALDEHYDE

Taurine dehydrogenase (TDH) was discovered by Kondo's group (Kondo *et al.*, 1971) and it is still referred to officially as EC 1.4.99.2, which indicates how difficult the enzyme is to study. Brüggemann et al. (2004) have now established that the physiological

electron acceptor is cytochrome *c*, so we presume that the enzyme may be reclassified as EC 1.4.2.- (Fig. 1). The membrane-bound enzyme has not been purified, and we have not yet confirmed our hypothesis that the *tauXY*-genes encode the structural proteins (Ruff *et al.*, 2003; Brüggemann *et al.*, 2004). Work is in progress to express the genes heterologously. In the meantime, we were forced to postulate that some taurine dehydrogenases (e.g. TauXY in *Rhodopseudomonas palustris*) require a native cytochrome *c* for activity, rather than bovine cytochrome *c* (Denger *et al.*, 2004b). Weinitschke has confirmed this idea by adding cytochrome *c*, which she isolated from *R. palustris*, to crude extract of *R. palustris*, and obtaining deamination of taurine (manuscript in preparation).

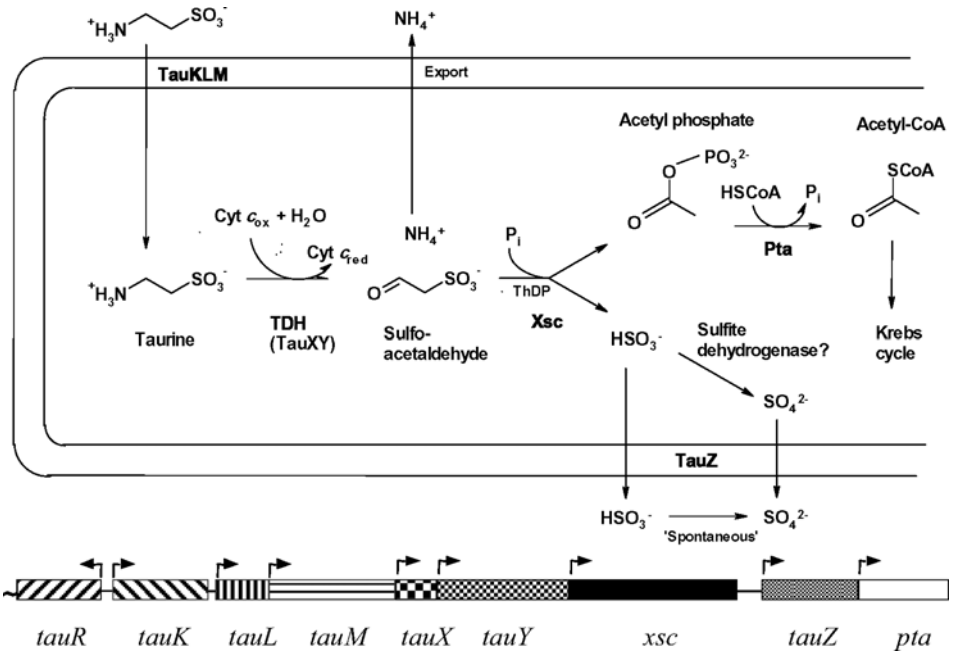

Figure 1. Presumed pathway for the dissimilation of taurine in *Paracoccus denitrificans* NKNIS, and the corresponding genes. Inducible TDH (taurine dehydrogenase), Xsc (sulfoacetaldehyde acetyltransferase) and Pta (phosphotransacetylase) have been assayed and *tauZ* is transcribed inducibly. The abbreviation ThDP represents thiamine diphosphate. The fate of acetyl-CoA is shown as the Krebs cycle, but this masks the requirement for anaplerotic enzymes, which in the β-Proteobacteria would be the glyoxylate shunt. The nature of the anaplerotic pathway in many α-Proteobacteria (e.g. strain NKNIS) is still unknown.

An alternative route to sulfoacetaldehyde is taurine:pyruvate transaminase (Tpa) [EC 2.6.1.77] coupled to alanine dehydrogenase (Ald) [EC 1.4.1.1] (Shimamoto and Berk, 1980). The enzymes were first purified from *Bilophila wadsworthia* and sequenced (Laue and Cook, 2000a,b). More recently, the enzymes were purified from *Rhodococcus* spp. and sequenced (Denger *et al.*, 2004a), but the organism in which the most complete pathway can be sketched is apparently *Silicibacter pomeroyi,* where it is derived from the

genome sequence (Moran *et al.*, 2004) (Fig. 2). Whereas there is a high degree of sequence homology amongst the TauXY sequences (Brüggemann *et al.*, 2004), there is considerable diversity amongst the Tpa sequences (not shown).

One organism, at least *Rhodobacter sphaeroides*, seems to express both the TDH and the Tpa, which can be deduced from the genome sequence (Novak *et al.*, 2004; Denger, 2005). Both reactions have been detected in *Paracoccus pantotrophus* as well (Mikosch *et al.*, 1999; Brüggemann *et al.*, 2004), but this has not yet been explored in detail.

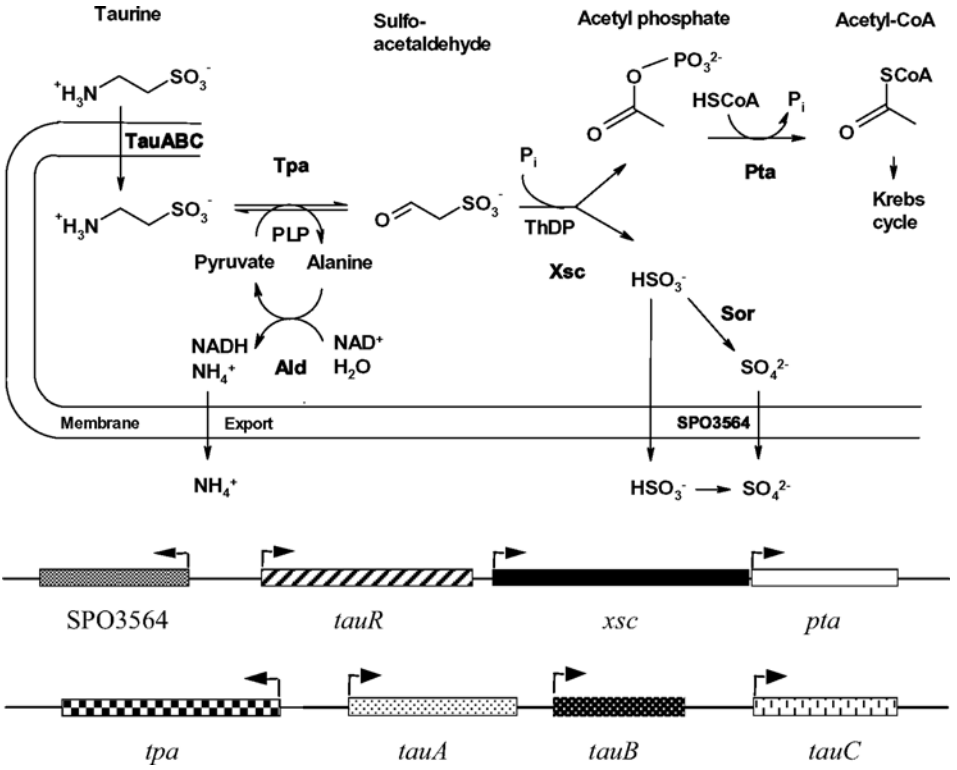

Figure 2. The presumed degradative pathway for taurine in *Silicibacter pomeroyi*^{T}, and the corresponding genes. This pathway is an example with an ABC transporter and a Tpa (taurine:pyruvate aminotransferase). Growth with taurine is quantitative (Denger, 2005). Inducible Ald (alanine dehydrogenase), Xsc (sulfoacetaldehyde acetyltransferase) and Sor (sulfite dehydrogenase) have been measured; neither Sor nor the *sor*-gene has been identified (Denger, 2005). The identity of the sulfate exporter (SPO3564) is hypothetical. The abbreviation ThDP represents thiamine diphosphate. The enzymes and transporters appear to be encoded by SPO0673-0676 (*tpa* to *tauC*), SPO3560-3562 (*pta* to *tauR*) and SPO0222 (*ald*).

4. SULFOACETALDEHYDE ACETYLTRANSFERASE

The nature of the desulfonation reaction was identified only recently and shown to be representative for enzymes studied previously (Ruff *et al.*, 2003). Sulfoacetaldehyde acetyltransferase (Xsc) [EC 2.3.3.15] is one of a newly recognized group of acetyltransferases in which the acetyl group is subject to isomerization during transfer.

Otherwise it seems to be a fairly standard thiamine diphosphate-coupled enzyme, which has the great advantage, especially for anaerobes, of yielding a high-energy bond, acetyl phosphate (Figs. 1 and 2). The sulfonate group is released as sulfite.

Several lines of evidence led us to hypothesize, and then to verify, this reaction. In part we discovered the phosphate-dependence of the reaction and in part the sequencing project yielded the neighbouring phosphotransacetylase gene (e.g. Figs. 1 and 2). Literature data pointed out the lability of acetyl phosphate under all sample work-up regimes used previously, so minor alterations in sampling brought dramatic changes in reactants, products and stoichiometry. The new reaction was established, a mechanism suggested (Cook and Denger, 2002) and the older version withdrawn by the Nomenclature Committee (NC-IUBMB).

The enzyme has been found in α-, β-, γ- and δ-Proteobacteria as well as in high- and low-G+C-content Gram-positive bacteria. Up till now we have recognized three subgroups of the enzyme, each of which has been purified and sequenced, and we suspect the presence of either another subgroup or an alternative enzyme type which is unstable (in e.g. *Bilophila wadsworthia*) (Ruff *et al.*, 2003; Brüggemann *et al.*, 2004).

5. PHOSPHOTRANSACETYLASE

The phosphotransacetylase (Pta) (phosphate acetyltransferase [EC 2.3.1.8]), which was first suggested from sequence data (see Chapter 4), could be detected as enzyme activity (Figs. 1 and 2) (Ruff *et al.*, 2003). As yet, direct confirmation has not been provided that the assayed enzyme is the product of the gene sequence indicated in Figs. 1 and 2. Preliminary analyses of sequenced genomes indicate that there are at least two classes of Pta. Indeed, in rare cases we have been unable to assay a phosphotransacetylase. The current hypothesis for the latter observation is that we have the wrong assay conditions, or that a class of Pta is unstable. This problem still needs to be addressed.

6. THE FATE OF TAURINE CARBON

Chapters 2-5 have given examples of aerobic dissimilation of taurine (e.g. the strict aerobe *Silicibacter pomeroyi* in Fig. 2). This is characterized by respiring about 50 % of the taurine to CO_2 and converting the other 50 % to biopolymers in cells. A similar situation holds true in Fig. 1, where the same dissimilative enzymes function whether *Paracoccus denitrificans* NKNIS is respiring with O_2 or with NO_3^- as the terminal electron acceptor. Under these conditions, the acetyl CoA will be processed via the Krebs cycle and an anaplerotic pathway. The latter is presumably the glyoxylate bypass in β-Proteobacteria, where the necessary genes are present in organisms with a sequenced genome (e.g. *Burkholderia xenovorans* LB400), or have been detected by direct assay (Denger and Cook, 2001). The nature of the anaplerotic pathway in several α-Proteobacteria is still unclear (Novak *et al.*, 2004).

A range of strictly anaerobic bacteria dissimilates taurine. There is a sulfite respiration in *Bilophila wadsworthia* and in several sulfate-reducing bacteria (Laue *et al.*, 1997; Lie *et al.*, 1998). There is one fermentation in *Desulfonispora thiosulfatigenes*, another in *Desulforhopalus singaporensis* (Denger *et al.*, 1999; Lie *et al.*, 1999). In all

cases, the major fate of the taurine carbon is acetate; some organisms utilize the remaining carbon for biosynthesis of biopolymers.

Where data are available, it would appear that taurine transaminase is used in these strict anaerobes to generate sulfoacetaldehyde. In very few cases has the presence of Xsc been confirmed (see Chapter 4). The enzyme that can be measured routinely in these organisms is Pta, but in this case it is accompanied by acetate kinase (Ack [EC 2.7.2.1]). We interpret this as conservation of energy by substrate level phosphorylation by Ack to yield ATP and the acetate, which is excreted by an unknown mechanism. Pta converts a portion of the acetyl phosphate generated by the often-putative Xsc to acetyl CoA for biosynthetic purposes.

7. THE FATE OF TAURINE NITROGEN DURING CARBON LIMITATION

When bacteria dissimilate taurine carbon, the ammonium ion is released (Figs. 1 and 2). This ammonium ion is in excess of requirements: bacteria require some 10 mol carbon per mol nitrogen. During growth, some 80% of the ammonium ion is recovered in the growth medium, usually concomitantly with growth, while the remainder is found in cell material (Denger *et al.*, 1997). The nature of the exporter is unknown, though an Amt protein (Khademi *et al.*, 2004) might be appropriate.

8. THE MANY FATES OF TAURINE SULFUR

The initial fate of taurine sulfur is always sulfite (Figs. 1 and 2), whether the organism is strictly anaerobic, facultatively anaerobic or strictly aerobic. The requirement of the cell for sulfur for biosynthetic purposes is negligible (about 1% of cell dry weight), so effectively all the sulfite remains to be processed.

The strictly anaerobic bacteria, which dissimilate taurine, apparently do so to obtain sulfite. These organisms then carry out a sulfite respiration via sulfite reductase (Laue *et al.*, 2001), and many of them excrete the sulfonate moiety as sulfide, although dismutation to sulfide and sulfate is known, as is the release of thiosulfate (Cook and Denger, 2002). In some ways, taurine can be considered as a non-toxic source of sulfite for these organisms (Laue *et al.*, 2001).

Sulfite can be regarded as a dual problem for facultative anaerobes and aerobes. In part there is the aspect of toxicity, and in part there is the problem of the osmotic pressure within the cell and the requirement to maintain constant conditions in the cell.

The problem of toxicity seems to be solved largely by oxidizing the sulfite to sulfate (Cook and Denger, 2002). This simple answer masks a range of problems. Is sulfite dehydrogenase [EC 1.8.2.1] or sulfite oxidase [EC 1.8.3.1] involved? There appears to be no report of sulfite oxidase [EC 1.8.3.1] in bacteria. And how many different sulfite dehydrogenases are there? The characterized bacterial sulfite dehydrogenase (SorAB) is periplasmic (Kappler *et al.*, 2000), which seems unsuitable to dispose of intracellular sulfite, and *sorAB*-like genes of high identity are not widespread in sequenced bacterial genomes. At least one other type of sulfite dehydrogenase is known (Reichenbecher *et al.*, 1999), and we believe this type to be present in *Silicibacter pomeroyi* (Denger, 2005), which does not contain the *sorAB*-genes. A lot of questions remain to be answered here.

The problem of homeostasis seems to be answered by sulfate exporters. We postulate that TauZ (Fig. 1) is a sulfate exporter (Rein *et al.*, 2005), one of many, but proof is still needed. We hypothesize that SPO3564 (Fig. 2) is another sulfate exporter, again without experimental evidence.

9. TAURINE AS A SOLE SOURCE OF NITROGEN FOR BACTERIA

It was clear from earlier work, e.g. Figs. 1 and 2, that taurine-nitrogen was used for growth. But what happens, should one supply a further source of carbon and allow the organism to utilize all the available nitrogen? We did this experiment with *Rhodococcus opacus*, and found that the complete dissimilative pathway for taurine was present and active (effectively as in Fig. 2), but that specific activity of the enzymes was reduced, presumably reflecting the lower requirement for nitrogen compared with carbon. No ammonium ion was released into the medium, so presumably the exporter was inactive (Denger *et al.*, 2004a).

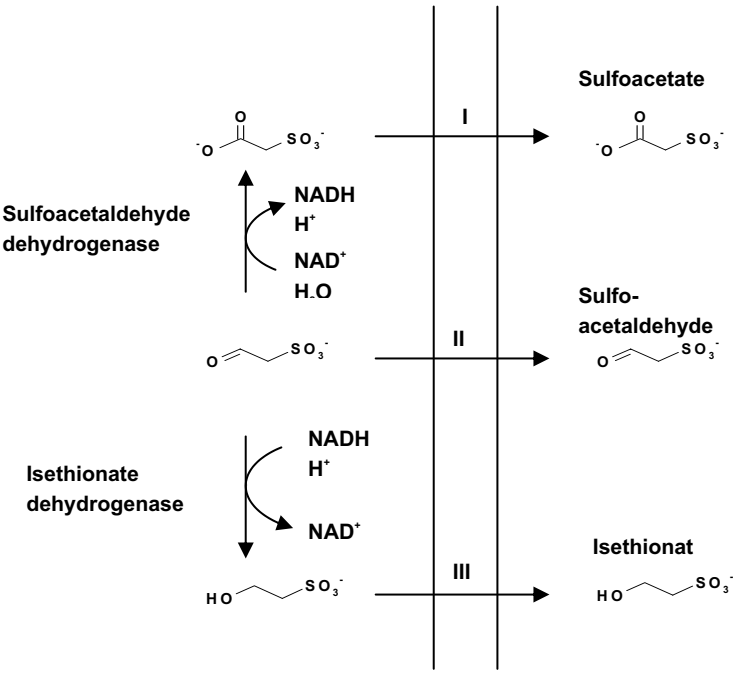

Figure 3. The major fates of carbon and sulfur when taurine is utilized as a sole source of nitrogen by different bacteria. Another fate is sulfate and CO_2, but that was minor in the experiments (Weinitschke *et al.*, 2005). *Rhodopseudomonas palustris* uses taurine dehydrogenase to generate sulfoacetaldehyde, sulfoacetaldehyde dehydrogenase to generate sulfoacetate and we hypothesize exporter I (Denger *et al.*, 2004b). *Acinetobacter calcoaceticus* also uses taurine dehydrogenase to generate sulfoacetaldehyde, which is excreted quantitatively by putative exporter II (Weinitschke *et al.*, 2005). *Klebsiella oxytoca* transaminates taurine to generate sulfoacetaldehyde, which is reduced by isethionate dehydrogenase, and the isethionate excreted by putative exporter III (Styp von Rekowski *et al.*, 2005).

Sequence data led us to test whether *Rhodopseudomonas palstris* utilized taurine nitrogen. It did so, but there was no excretion of sulfate, in contrast to the metabolism of *Rhodococcus opacus*. Instead, the organism excreted sulfoacetate quantitatively (Fig. 3). We thus had a new pathway to generate sulfoacetate, which was previously known only from the degradation of the plant sulfolipid (Denger *et al.*, 2004b).

Further exploration of this phenomenon showed that the release of an organosulfonate from taurine under these conditions was normal; only about 10% of isolates released sulfate (Weinitschke *et al.*, 2005). One of the products formed was sulfoacetaldehyde: as the compound is utilized as a growth substrate by other bacteria (Lie *et al.*, 1996), we presume that the excretion of sulfoacetaldehyde is not unusual.

The third organosulfonate that we discovered in quantitative amounts was isethionate (Styp von Rekowski *et al.*, 2005). The generation of isethionate from taurine in faecal material was known (Fellman *et al.*, 1980), and those authors attribute mammalian isethionate to bacterial production in the gut. We now supply a physiological and biochemical background to that observation.

Just as the excretion of sulfate requires an exporter, in our hypotheses (Chapter 8), we see a requirement for exporters of sulfoacetate, sulfoacetaldehyde and isethionate (Fig. 3). In the latter cases, however, there is even less experimental evidence that in Chapter 8.

10. REGULATION OF INDUCTION

The utilization of taurine described in Chapters 2-9 involves regulation of enzyme induction. In almost all cases where we have data, a gene neighbouring a region encoding a recognized portion of the pathways in Figs. 1-3 is found in common. We have termed it the *tauR*-gene (Figs. 1 and 2), because of its similarity to known transcriptional regulators (Ruff *et al.*, 2003; Brüggemann *et al.*, 2004), but here again, we have not yet tested the hypothesis. The genome of *Desulfotalea psychrophila* (Rabus *et al.*, 2004), with candidates for *tauKLM*, *tpa*, ald and *xsc* (see Figs. 1 and 2), contains no *tauR*-like gene, so a different regulatory protein or mechanism seems likely.

The available evidence indicates that sulfite dehydrogenase is inducible. When an organism utilizes more than one inducible desulfonation pathway (e.g. *Paracoccus pantotrophus* NKNCYSA), the sulfite dehydrogenase is induced in both cases (Rein *et al.*, 2005), which shows that the regulation of sulfite dehydrogenase is independent of the regulation of the degradation of sulfonates.

11. SCAVENGING FOR SULFUR UNDER GLOBAL REGULATION

The requirement for sulfur for biomolecules is orders of magnitude lower than for e.g. carbon (Chapter 8). Correspondingly, different enzymes are needed under the different conditions of sulfur limitation and carbon limitation. Similarly, different regulation is required. Kertesz (2000) describes this in detail.

The regulation is not the specific induction presumed in Chapter 10. Instead, there is global regulation, whereby the cell under sulfate starvation simultaneously switches on all scavenging systems it contains; sometimes, additional regulatory circuits are involved. One of those is for taurine.

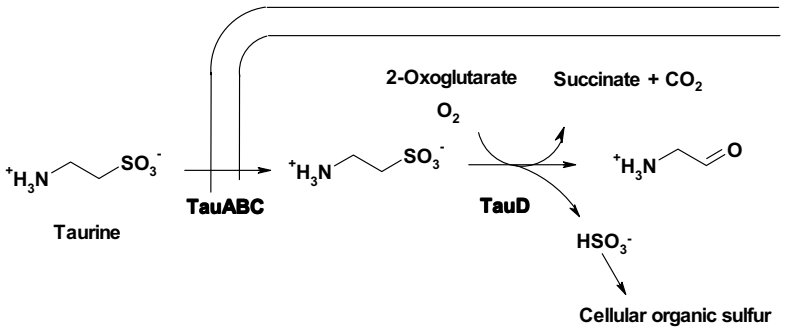

Figure 4. Desulfonation of taurine involving the products of the taurine cluster *tauABCD*, which was initially discovered in *Escherichia coli* (Eichhorn *et al.*, 1997, 2000).

The taurine cluster contains four genes, *tauABCD*. The transporter, TauABC (Fig. 4), was introduced earlier (Chapter 2). The desulfonation is oxygenolytic (Fig. 4). Taurine dioxygenase (TauD [EC 1.14.11.17]) is a 2-oxoglutarate-dependent oxygenase, which generates sulfite, succinate and 2-aminoacetaldehyde. This system is very widespread in bacteria, as a search of the NCBI database with the BLAST algorithm and any of the protein sequences shows.

12. FINAL COMMENTS

The degradative path for taurine is short, but it includes novel biochemistry and multiple transport and regulatory phenomena of general relevance, which are poorly understood and which we hope to elucidate.

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THE REACTIVITY OF HYPOTAURINE AND CYSTEINE SULFINIC ACID WITH PEROXYNITRITE*

Mario Fontana, Silvestro Duprè, and Laura Pecci**

1. INTRODUCTION

The oxidation of the sulfinic group of both hypotaurine and cysteine sulfinic acid with production of the respective sulfonate, taurine and cysteic acid is a crucial point for the generation of taurine in mammalian tissues (Wright *et al*., 1986; Huxtable, 1992). It has been proposed that the high levels of taurine found in tissues or cells such as sperm, neutrophils and retinal tissue (Pasantes-Morales *et al*., 1972; Alvarez and Storey, 1983; Learn *et al*., 1990; Green *et al*., 1991; Holmes *et al*., 1992) would reflect the turnover of hypotaurine *via* oxidative reactions and might be viewed as an indirect measure of the oxidative stress associated with such tissues. However, the mechanism of the oxidative reaction of the sulfinic group is not yet clearly defined. Recently, it has been shown that, besides nonspecific oxidants such as UV irradiation, hypochlorite, hydroxyl radical and photochemically generated singlet oxygen, also peroxynitrite mediates the oxidation of both hypotaurine and cysteine sulfinic acid to taurine and cysteic acid, respectively (Ricci *et al*., 1978; Green *et al*., 1985; Fellman *et al*., 1987; Pecci *et al*., 1999; Fontana *et al*., 2005). These findings have been related to the proposed role of hypotaurine as an antioxidant and free radical trapping agent *in vivo* (Aruoma *et al*., 1988; Tadolini *et al*., 1995). According to this, hypotaurine and cysteine sulfinic acid are able to prevent peroxynitrite-mediated reactions such as tyrosine nitration, α_1-antiproteinase inactivation and low-density lipoprotein oxidative modification (Fontana *et al*., 2004).

Peroxynitrite is a strong oxidizing and nitrating agent, which can be produced by the reaction of nitric oxide with superoxide anion (Koppenol *et al*., 1992; Huie and Padmaja, 1993; Pryor and Squadrito, 1995) and represents a reactive toxic species that can mediate cellular and tissue damage in various human diseases, including neurodegenerative disorders, inflammatory and autoimmune diseases (Eiserich *et al*., 1998; Stewart and Heales, 2003). At physiological pH both peroxynitrite anion ($ONOO^-$) and its conjugate

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* Dedicated to Professor Doriano Cavallini (1916-2005).

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Taurine 6
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acid (ONOOH, $pK_a = 6.8$) are present. Peroxynitrite is quite stable but upon protonation to peroxynitrous acid; it decays rapidly ($t_{1/2} < 1$ s) generating nitrate together with highly oxidizing and nitrating reactive species. It has been reported that peroxynitrite can oxidize suitable substrates, either through a direct one- or two-electron mechanism or by an indirect one-electron reaction involving hydroxyl ($^{\bullet}OH$) and nitrogen dioxide ($^{\bullet}NO_2$) radicals released during peroxynitrite homolysis (Radi *et al*., 2001).

In a recent work, the reaction of the sulfinates, hypotaurine and cysteine sulfinic acid with peroxynitrite has been shown to be associated with extensive oxygen uptake, suggesting that hypotaurine and cysteine sulfinic acid are oxidized by one-electron transfer mechanism to sulfonyl radicals which are converted to sulfonates by further oxygen-dependent reactions (Fontana *et al*., 2005). Beside the one-electron mechanism, hypotaurine and cysteine sulfinic acid can be oxidized by the two-electron pathway leading to direct sulfonate formation without oxygen consumption. The oxidation of sulfinates by peroxynitrite may thereby occur via the two reaction pathways.

In order to evaluate the mechanisms of oxidation of sulfinates by peroxynitrite and the relevance of the one- and the two-electron oxidative pathways, we compared oxygen consumption and sulfonate production at various concentrations of the two sulfinates. Peroxynitrite decomposition produces nitrate as main product while after reaction with a target molecule nitrite, whose quantity depends on the pathway of the oxidative reaction, is formed (Kissner and Koppenol, 2002; Jourd'heuil *et al*., 2003). Therefore, the amount of nitrite and nitrate formed in the reaction of peroxynitrite with hypotaurine and cysteine sulfinic acid at different pH has been also determined.

2. MATERIALS AND METHODS

2.1. Chemicals

Cysteine sulfinic acid, cysteic acid, hypotaurine, taurine were obtained from Sigma Chem Co. Diethylenetriamine pentaacetic acid (DTPA), tetrabutylammonium bisulfate, *o*-phthaldialdehyde and manganese dioxide were from Fluka. All other reagents were of the highest purity commercially available.

Peroxynitrite was synthesized from potassium nitrite and hydrogen peroxide under acidic conditions as previously described (Beckman *et al*., 1994), and excess hydrogen peroxide was removed by treatment with granular manganese dioxide. Typical peroxynitrite concentration after freeze fractionation was 600-700 mM as determined by absorbance at 302 nm using a molar absorption coefficient of 1670 $M^{-1}cm^{-1}$. Stock solutions of peroxynitrite were diluted with 0.1 M NaOH immediately before use to achieve the desired concentration.

2.2. Reaction of Hypotaurine or Cysteine Sulfinic Acid With Peroxynitrite

The reaction mixture contained hypotaurine or cysteine sulfinic acid at appropriate concentrations in 0.2 M phosphate buffer at pH 7.4 or 5.5. To avoid metal-catalyzed oxidative reactions, all samples contained 0.1 mM DTPA. The reaction was started by addition of peroxynitrite at a final concentration of 0.2 mM. To control for nonspecific effects of contaminating substances present in the peroxynitrite solutions or to stable peroxynitrite-decomposition products (nitrite and nitrate), peroxynitrite was first incubated

in phosphate buffer/DTPA for 10 min before the addition of hypotaurine or cysteine sulfinic acid (reverse order addition).

2.3. Oxygen Uptake

Oxygen uptake was performed using a Gilson 5/6 oxygraph and measured with a Clark type electrode in a water-jacketed cell (1.8 ml) at 25°C. The saturation oxygen concentration at this temperature was taken as 235 μM.

2.4. HPLC Analysis

Hypotaurine, cysteine sulfinic acid, taurine and cysteic acid were determined by high performance liquid chromatography (HPLC) using the *o*-phthaldialdehyde reagent (Hirschberger *et al*., 1985). Analyses were carried out with a Waters Chromatograph equipped with a Perkin-Elmer model LS-1 LC fluorescence detector using a 340-nm filter for excitation with an emission wavelength of 450 nm. The column was a 250 x 4.6 mm I.D. Simmetry C_{18}, 5 μm (Waters). The mobile phases were (A) 0.05 M sodium acetate (pH 5.5)-metanol (80:20, v/v) and (B) 0.05 M sodium acetate (pH 5.5)-metanol (20:80, v/v). The elution gradient was linear from A to 50% B in 5 min followed by isocratic at 50% B. Flow rate was 1 ml/min at room temperature. The elution times of cysteic acid, cysteine sulfinic acid, taurine and hypotaurine were 7.5, 10.5, 24.5, and 26 min, respectively.

Nitrite and nitrate were analyzed by ion-pairing HPLC as described previously (Jourd'heuil *et al*., 2003). Samples were injected onto a 250 x 4.6 mm I.D. Atlantis C_{18}, 5 μm (Waters) isocratically running at a flow rate of 1 ml/min with 10 mM K_2HPO_4, 10 mM tetrabutylammonium bisulfate in water-acetonitrile (95:5, v/v, pH 7). Detection was made at 210 nm using a Waters 996 photodiode array detector. The elution times of nitrite and nitrate were 8.5 and 20.5 min, respectively.

2.5. Statistical Analysis

Results are expressed as mean values ± SEM of at least three separate experiments. Graphics and data analysis were performed using GraphPad Prism 4 software.

3. RESULTS AND DISCUSSION

3.1. Peroxynitrite-Mediated Oxidation of Hypotaurine and Cysteine Sulfinic Acid

The oxidation of the sulfinates, hypotaurine and cysteine sulfinic acid, by peroxynitrite has been evaluated by monitoring the oxygen consumption and the production of the corresponding sulfonates, taurine and cysteic acid, at physiological pH.

When peroxynitrite is added to a solution containing the sulfinates (RSO_2^-), fast oxygen consumption is observed, suggesting the generation of intermediate radicals which react with oxygen. It is therefore proposed that the peroxynitrite-mediated oxidation of sulfinates probably involves an initial one-electron transfer mechanism with generation of sulfonyl radical ($RSO_2^{\bullet}$). Despite the controversial aspect of peroxynitrite chemistry, presently most investigators agree that one-electron oxidation is not a direct

reaction of peroxynitrite but depends on the interaction of the target molecule with the nitrogen dioxide ($^{\bullet}NO_2$) and hydroxyl radicals ($^{\bullet}OH$) released during the degradation process of peroxynitrite (Radi *et al*., 2001). According to this, the sulfinates can be indirectly oxidized to sulfonyl radicals by the peroxynitrite-derived free radicals:

$$RSO_2^- + {}^{\bullet}OH/{}^{\bullet}NO_2 \rightarrow RSO_2^{\bullet} + OH^-/NO_2^- \quad (1)$$

HPLC analyses of the incubation mixtures at the end of the reaction show that hypotaurine and cysteine sulfinic acid are oxidized to the corresponding sulfonates, taurine and cysteic acid.

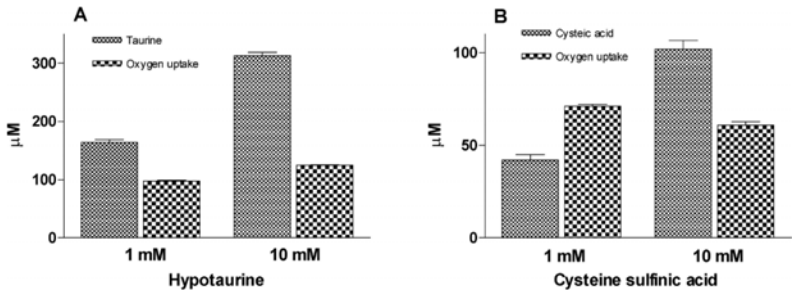


Figure 1. Oxidation of hypotaurine and cysteine sulfinic acid by peroxynitrite at pH 7.4. Peroxynitrite (200 µM) was added into the oxygraph chamber at 25°C, containing (1 or 10 mM) hypotaurine (A) or cysteine sulfinic acid (B) in 0.2 M phosphate buffer and 0.1 mM DTPA, pH 7.4, and the O_2 consumption was recorded. The reaction mixtures were subsequently analyzed by HPLC.

Fig. 1A shows oxygen uptake and the amount of taurine produced after the addition of 200 µM peroxynitrite to 1 mM and 10 mM hypotaurine at pH 7.4. It can be observed that at 10 mM hypotaurine, the amount of taurine produced is higher than the concentration of peroxynitrite added, evidencing the occurrence of a chain reaction mechanism responsible of amplification of the oxidation. Furthermore, the results show that the amount of oxygen consumed per mol of sulfonate produced is much lower at 10 mM hypotaurine.

Fig. 1B shows oxygen uptake and the amount of cysteic acid produced by reacting cysteine sulfinic acid with peroxynitrite in the same experimental condition as above. It can be observed: (a) at 1 mM cysteine sulfinc acid concentration, the amount of oxygen uptake is much higher than cysteic acid produced; (b) at 10 mM cysteine sulfinic acid, the amount of oxygen uptake is lower than at 1 mM cysteine sulfinic acid; and (c) the yields of cysteic acid are lower when compared to taurine obtained after the reaction of peroxynitrite with hypotaurine under the same reaction conditions. These results can be explained by the already reported tendency of cysteine sulfinic acid-derived radical to decompose sulfur dioxide (SO_2) produced and a carbon-centered radical ($R^{\bullet}$) (Harman *et al*., 1984). Subsequent oxidation of sulfite (aqueous sulfur dioxide) to sulfate involves additional free radical mechanisms leading to oxygen consumption (Mottley and Mason, 1988; Karoui *et al*., 1996). The previously reported detection of sulfate in the incubation mixtures of cysteine sulfinic acid with peroxynitrite indicates that cysteine sulfinic acid-

derived sulfonyl radical undergoes a significative decomposition at physiological pH 7.4 (Fontana *et al*., 2005). Accordingly, the high oxygen uptake, observed during the reaction of cysteine sulfinic acid with peroxynitrite, can account, in addition to that required for oxidation of cysteine sulfinic acid to cysteic acid, for the oxidation of sulfite to sulfate. It is also possible that the high reactive alkyl radical ($R^{\bullet}$) can react with oxygen, contributing to the observed oxygen uptake. Interestingly, compared to the cysteine sulfinic acid-derived sulfonyl radical, the sulfonyl radical derived from the oxidation of hypotaurine appears to have a much lower tendency to decompose as indicated by the finding that the amount of oxygen consumed per mole of taurine produced is much lower than that observed in the oxidation of cysteine sulfinic acid.

The oxidative reactions mediated by peroxynitrite are expected to take place by both one- and two-electron mechanisms (Radi *et al*., 2001). As mentioned above, it is recognized that the one-electron mechanism is not a direct reaction of peroxynitrite but depends on the radicals $^{\bullet}OH$ and $^{\bullet}NO_2$ derived from peroxynitrite decomposition. In the two-electron mechanism, peroxynitrite reacts directly with the target molecule in an overall second-order process. Although the observed oxygen consumption associated with oxidation of sulfinates by peroxynitrite indicates a relevant contribution of the one-electron pathway, the two-electron mechanism could represent an additional route of oxidation of sulfinates leading to direct sulfonate formation without oxygen consumption.

The results that the oxygen consumed per mole of sulfonate produced decreases considerably with the increase of sulfinate concentration, indicate that the reaction may occur *via* the two mechanisms whose relative importance depends on reagent concentrations. At 1 mM RSO_2^- concentration, where the oxygen uptake associated with the oxidation of sulfinates is higher; the one-electron mechanism with the intermediate formation of sulfonyl radicals is likely to predominate. In this case, the radicals $^{\bullet}OH$ and $^{\bullet}NO_2$ produced during peroxynitrite decomposition oxidize RSO_2^- to $RSO_2^{\bullet}$ radicals which are responsible of oxygen consumption. Additionally, the formation of sulfonyl radicals initiates an oxygen-dependent radical chain reaction that could greatly amplify the importance of the one-electron pathway. At 10 mM RSO_2^- concentrations the oxygen uptake associated with the oxidation of sulfinates decreases, as the second-order reaction of the two sulfinates with peroxynitrite becomes more significant. In this pathway, peroxynitrite participates as two-electron oxidant and will oxidize the sulfinates without the formation of sulfonyl radicals, and thus with no associated oxygen consumption (see Scheme 1).

3.2. Effect of pH on the Interaction of Peroxynitrite With Sulfinates

To evaluate the effect of pH on peroxynitrite-mediated oxidation of sulfinates, we monitored hypotaurine and cysteine sulfinic acid oxidation at pH 5.5 by sulfonate production and oxygen consumption. The data presented in Fig. 2 show that in the reaction of 1 mM sulfinates with 200 μM peroxynitrite at acidic pH, the yields of representive sulfonates are greater than those at pH 7.4. These data suggest that peroxynitrous acid (ONOOH, pK_a = 6.8) is the reactive species. Kinetic experiments performed at various pH are consistent with this interpretation (Fontana *et al*., 2005).

The oxygen uptake associated with the reaction carried out at pH 5.5 reveals a stoichiometry of approximately 0.5 mol of oxygen consumed per mol of sulfonate produced. These results indicate that at acidic pH, the cysteine sulfinic acid-derived sulfonyl radical appears to have a lower tendency to decompose.

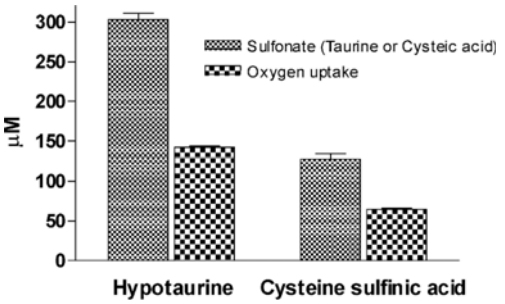


Figure 2. Oxidation of hypotaurine and cysteine sulfinic acid by peroxynitrite at pH 5.5. Peroxynitrite (200 µM) was added into the oxygraph chamber at 25°C, containing 1 mM hypotaurine (A) or 1 mM cysteine sulfinic acid (B) in 0.2 M phosphate buffer and 0.1 mM DTPA, pH 5.5, and the O_2 consumption was recorded. The reaction mixtures were subsequently analyzed by HPLC.

3.3. Measurement of Nitrite and Nitrate During the Oxidation of Sulfinates by Peroxynitrite

In the two-electron process of oxidation of sulfinate by peroxynitrite, nitrite and sulfonate would be the only products:

$$RSO_2^- + ONOO^-/ONOOH \rightarrow RSO_3^- + NO_2^- \quad (2)$$

If sulfinates are oxidized by one-electron mechanism, also nitrate will be formed. This is because the peroxynitrite-derived radicals $^{\bullet}OH$ and $^{\bullet}NO_2$, initially formed in a solvent cage, undergo rapid recombination to form nitrate (about 70%) or escape the cage (about 30%) to give free radicals, which react with sulfinates (Mottley and Mason, 1988; Karoui *et al.,* 1996). Thus, the product distribution of nitrite and nitrate can provide a further mean to establish the contribution of the two pathways.

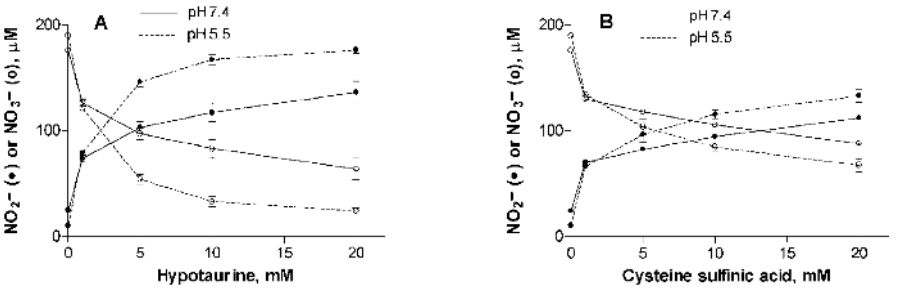
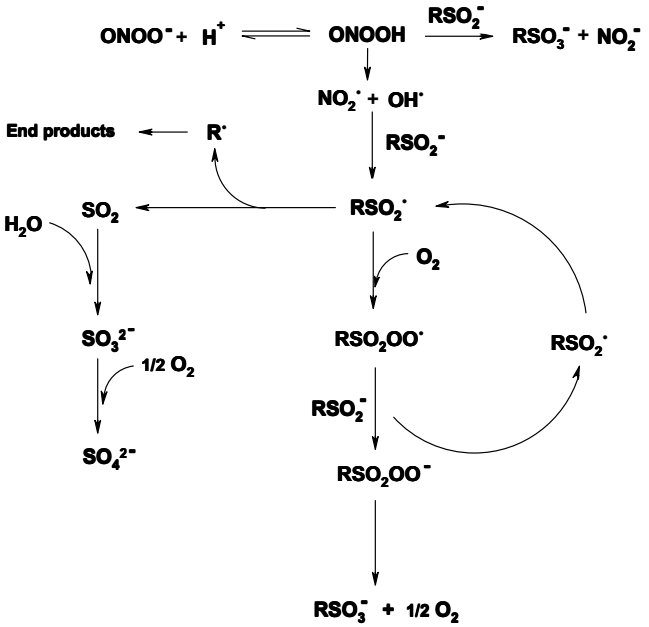


Figure 3. Oxidation of hypotaurine and cysteine sulfinic acid by peroxynitrite: nitrite and nitrate formation. Peroxynitrite (200 µM) was incubated with different concentrations of hypotaurine (A) and cysteine sulfinic acid (B), in 0.2 M phosphate buffer containing 0.1 mM DTPA at room temperature for 15 min, followed by the determination of NO_2^- and NO_3^-.

The concentrations of nitrite and nitrate formed during the decomposition of the peroxynitrite at pH 7.4 and 5.5 in the presence of various concentrations of sulfinates are reported in Fig. 3. The increase of nitrite and the decrease of nitrate formed during the reaction of 200 μM peroxynitrite with increasing concentrations of sulfinates further support the conclusion that the two pathways coexist and that the direct reaction (i.e., two-electron oxidation) would prevail quantitatively over the one-electron oxidation when sulfinates are present in large excess over peroxynitrite. The unexpected low yield of nitrite observed in the reaction of peroxynitrite with cysteine sulfinic acid could be explained by a partial reoxidation of produced nitrite by secondary radicals generated by decomposition of the cysteine sulfinic acid-derived sulfonyl radicals.

4. CONCLUSION

The data presented in this work demonstrate that the sulfinates (RSO_2^-), hypotaurine and cysteine sulfinic acid are oxidized by peroxynitrite to form the corresponding sulfonates (RSO_3^-), taurine and cysteic acid. The data demonstrate that the peroxynitrite-mediated oxidation of sulfinates may occur either through one- or two-electron pathways whose relative importance depends on reagent concentrations and pH. We propose that one-electron oxidation, mediated by the peroxynitrite-derived free radicals, produces sulfonyl radicals ($RSO_2^{\bullet}$) as intermediates. The peroxynitrite-mediated oxidative pathways of sulfinates are shown in the scheme 1.



Scheme 1. Oxidative pathways of sulfinates.

The consumption of oxygen by the peroxynitrite-dependent oxidation of sulfinates could result from the known reaction of sulfonyl radicals with oxygen with production of sulfonyl peroxyl radical ($RSO_2OO^{\bullet}$) (Sevilla *et al*., 1990). The sulfonyl peroxyl radical is a highly reactive intermediate (Sevilla *et al*., 1990) and its possible reaction with excess sulfinate can proceed to give peroxysulfonate (RSO_2OO^{-}). The peroxysulfonate formed would decompose to give sulfonate and molecular oxygen.

It should be noted that sulfonyl radicals, as shown in the scheme, may initiate an oxygen-dependent radical chain propagation step that could be responsible of amplification of the oxidation.

Other possible reactions previously suggested for sulfonyl radical (Fellman *et al*., 1987; Green and Fellman, 1994) include its dimerization to form the corresponding disulfone ($RSO_2–SO_2R$) and its condensation with the sulfonyl peroxyl radical intermediate leading to the persulfonate ($RSO_2–OO–SO_2R$). Subsequent hydrolysis of the disulfone or of the persulfonate could represent additional routes for the production of sulfonates:

$$RSO_2–SO_2R + H_2O \rightarrow RSO_2^{-} + RSO_3^{-} + 2H^{+} \quad (3)$$

$$RSO_2–OO–SO_2R + H_2O \rightarrow 2\,RSO_3^{-} + ½\,O_2 + 2H^{+} \quad (4)$$

However, the production of sulfonates through the intermediate formation of disulfone do not require oxygen, in contrast with the observed oxygen consumption associated with the oxidative reaction.

In conclusion, the above results indicate that peroxynitrite and its derived species can be included into the non-specific biological oxidant able to accomplish the oxidation of the sulfinic group of hypotaurine and cysteine sulfinic acid to the sulfonic of taurine and cysteic acid, respectively. However, the formation of intermediate sulfonyl radicals, which can propagate oxidative reactions, raises the question about the metabolic fate and/or the pathophysiological significance of these species. Among sulfur-centered radicals, it has been already shown that thiyl radicals ($RS^{\bullet}$), generated by one-electron oxidation of thiols, react with molecular oxygen to form thiyl peroxyl radical ($RSOO^{\bullet}$) which can rearrange to sulfonyl radical ($RSO_2^{\bullet}$) that further react with oxygen to generate the sulfonyl peroxyl radical ($RSO_2OO^{\bullet}$) (Sevilla *et al*., 1990). Both thiyl and thiyl-derived radicals such as sulfonyl radical are potent initiators of lipid peroxidation (Schöneich *et al*., 1992), thus behaving as oxidants, which can exert damaging effects *in vivo*. However, the biological relevance of $RS^{\bullet}$-derived radicals remains a matter of debate and recently an additional group of redox active molecules termed reactive sulfur species (RSS) has been proposed to be formed *in vivo* under conditions of oxidative stress (Giles *et al*., 2001). Although sulfur-containing molecules are generally considered to act as antioxidants and, in particular, our previous studies showed that hypotaurine and cysteine sulfinic acid have the ability to inhibit peroxynitrite-dependent reactions (Fontana *et al*., 2004), the transient formation of sulfur reactive species during the oxidative reaction, could have a physiological importance which remain to be investigated.

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CYSTEAMINE DIOXYGENASE: EVIDENCE FOR THE PHYSIOLOGICAL CONVERSION OF CYSTEAMINE TO HYPOTAURINE IN RAT AND MOUSE TISSUES

Relicardo M. Coloso, Lawrence L. Hirschberger, John E. Dominy, Jeong-In Lee, and Martha H. Stipanuk*

1. INTRODUCTION

During the 1960s, Cavallini and coworkers demonstrated the presence of a protein with cysteamine dioxygenase activity in animal tissues and proposed that this enzyme was important in the conversion of cysteamine to hypotaurine (Cavallini *et al.,*1963, 1966). Based on the earlier demonstration that cysteamine was a component of coenzyme A (Baddiley *et al*., 1953; Novelli *et al*., 1954) as well as their own work, they proposed a coenzyme A-cysteamine pathway for taurine production. As shown in Fig. 1, coenzyme A is synthesized using cysteine, but the cysteinyl moiety is subsequently decarboxylated to produce the cysteamine moiety of coenzyme A. Coenzyme A turnover results in production of pantetheine, and pantetheine is hydrolyzed by pantetheinase to yield cysteamine and pantothenic acid. The released cysteamine is oxidized to hypotaurine and then further oxidized to taurine by an unknown mechanism. An alternative pathway of taurine synthesis from cysteine is also shown in Fig. 1. This pathway involves the oxidation of cysteine to cysteinesulfinate, the decarboxylation of cysteinesulfinate to hypotaurine, and the conversion of hypotaurine to taurine.

After the association of low cysteinesulfinate decarboxylase activity, and subsequently of low cysteine dioxygenase activity, with a limited capacity of cats and certain other species to synthesize taurine (Knopf *et al.*, 1978; Stipanuk *et al*., 1992, 1994) the focus of research in taurine biosynthesis moved away from the cysteamine pathway to the cysteinesulfinate pathway and remained there for several decades (Stipanuk *et al.*, 1986, 2004). However, identification of a lack of panthothenate kinase (PANK2) as the basis for Hallervorden-Spatz syndrome (now known as neurodegeneration with brain iron accumulation or pantothenate-kinase associated neurodegeneration) (Zhou *et al*., 2001) and the discovery that *Vanin-1* is a membrane-bound pantetheinase that cleaves pantetheine to cysteamine and pantothenic acid (Pitari

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et al., 2000) have renewed interest in cysteamine as a potentially significant intermediate in taurine biosynthesis. Pantothenate kinase is necessary for the synthesis of coenzyme A from cysteine, and pantetheinase is necessary for the release of the cysteamine moiety derived from cysteine during coenzyme A degradation.

The high cysteine concentration in some regions of the brain of PANK2-deficient patients (Perry *et al*., 1985) suggests that coenzyme A synthesis may play a quantitatively important role in cysteine removal in some tissues. However, no direct measurements of cysteine utilization for coenzyme A synthesis or of coenzyme A turnover on a whole body level have been reported and little information is available for particular tissues or cell types. Perfusion of rat heart with 7 μM pantothenic acid and 10 μM [^{35}S]cysteine (and mercaptodextran to help maintain cysteine in the thiol form) resulted in 1.6-times as much radioactivity being incorporated into coenzyme A and coenzyme A intermediates (e.g., 4'-phosphopantetheine, dephosphocoenzyme A) as into protein (Chua *et al*., 1984).

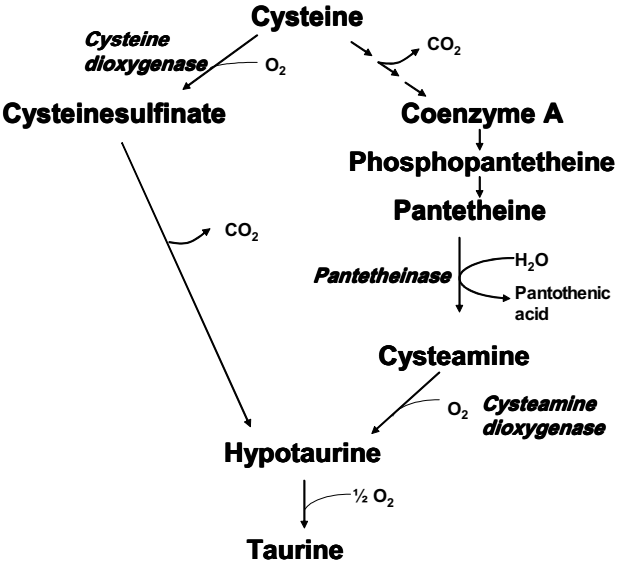

Figure 1. Alternative pathways for taurine production.

Pantetheine is formed in the process of coenzyme A degradation (see Fig. 1). Pantetheinase activity has been found in many tissues from mammals but its biological role has never been explored in detail. The pig kidney enzyme was purified and biochemically characterized in the 1970s by Duprè and Cavallini (1979). The mouse *Vanin-1* was initially characterized as a membrane molecule expressed by a subset of thymic stromal cells that was involved in the homing of bone marrow precursor cells into the thymus (Aurrand-Lions *et al.*, 1996). Then, partial sequences of the pig pantetheinase were reported (Maras *et al*., 1999), and on the basis of sequence similarities, the identity between pantetheinase and mouse *Vanin-1* was postulated (Pitari *et al.*, 2000). Pitari *et al.* (2000) went on to demonstrate that the *Vanin-1* gene encodes a pantetheinase that is widely expressed in mouse tissues. They showed that pantetheinase activity is

specifically expressed by *Vanin-1* transfected cells and is immuno-depleted by specific antibodies; that *Vanin-1* is a GPI-anchored pantetheinase (an ectoenzyme); and that *Vanin-1* null mice are deficient in membrane-bound pantetheinase activity in kidney and liver. Furthermore, they showed that a major consequence of disruption of the *Vanin-1* gene in mice is that liver and kidney of these mice were depleted of detectable free cysteamine; cysteamine levels in liver and kidney of wild-type mice were 24 and 15 nmol/g, respectively. Thus, the membrane-bound pantetheinase appears to be the major source of cysteamine in tissues under physiological conditions.

Evidence for a quantitatively significant rate of coenzyme A synthesis in brain and heart and for significant cysteamine production during coenzyme A degradation in liver or kidney indicate the need to further consider the relative importance of dietary cysteine as a precursor for coenzyme A synthesis and also the relative importance of cysteamine as a precursor for taurine biosynthesis. This paper summarizes several approaches which our laboratory has taken recently in an effort to further explore the role of cysteamine in taurine biosynthesis.

2. MATERIALS AND METHODS

2.1. Measurement of Cysteamine Dioxygenase in Rat and Mice Tissues

Tissues were obtained from adult mice (C57/B6) or rats (Sprague-Dawley). Mice were anesthetized with CO_2 and rats were anesthetized with pentobarbital prior to tissue collection. The liver, kidney and brain were removed immediately, frozen in liquid nitrogen and stored at -80^{o}C until used. Minced liver, kidney or brain was homogenized in 2.5 volumes of 0.01 M potassium phosphate buffer, pH 7.6. The homogenate was sonicated by four 15 s bursts (15 s cooling period between bursts) and centrifuged at 1800xg for 10 min at 4^{o}C; the supernatant was used for the enzyme assay.

The assay procedure for cysteamine dioxygenase was modified from the original method used by Cavallini *et al*. (1966). A 250-µl aliquot of supernatant prepared as previously described was pipetted into an Eppendorf tube containing 25 µl of 1 M potassium phosphate buffer, pH 7.6, 250 µl of 10 mM cysteamine, 50 µl of 50 mM sodium sulfide, and 175 µl of deionized water. The final cysteamine concentration in the assay mixture was 3.3 mM. The tube was then placed into a Thermomixer (Eppendorf AG, Hamburg, Germany) at 37^{o}C. Incubations were terminated at 1 h by the addition of 250 µl of 5% (w/v) sulfosalicylic acid. Zero-time incubations served as blanks. The mixture was then centrifuged at 10,620xg in an Eppendorf centrifuge 5810 R (Eppendorf, AG, Hamburg, Germany) for 10 min at 4^{o}C. The acid supernatant was decanted into a clean tube and stored at -20^{o}C until hypotaurine and taurine were measured. Enzyme activity was expressed either as µmol hypotaurine produced per h per g wet wt of tissue or as nmol hypotaurine produced per h per mg of protein.

2.2. Assay of Tissue Protein Content

Total protein in tissue homogenates was assayed by the bicinchoninic acid method of Smith *et al.* (1985) using bovine serum albumin as a standard. The total protein was used

as a basis for expression enzyme activity.

2.3. Dietary Study

Male Sprague-Dawley rats were randomly assigned to groups of 3. Each group of 3 rats was housed together in one cage in a room that was dark from 06:00 to 18:00 h. Food was placed in the cage at the beginning of the dark period and removed at the end of the dark period each day to accustomize rats to beginning to eat at the beginning of the dark period. Rats were initially given a high protein diet (400 g casein/kg diet) for 7 days. At the beginning of the dark period on the experimental day, rats were given a new diet: low protein (100 g casein/kg diet); low protein + cysteamine (100 g casein + 7.2 g cysteamine/kg diet); or low protein + cysteine (100 g casein + 8.1 g cysteine/kg diet). Diets were prepared by mixing 100 g of diet with 100 ml of hot 3% (w/v) agar solution. One group of rats on each diet was killed at 12:00 h (6 hours after introduction of the new diet), and one group of rats on each diet was killed at 16:00 h (10 hours after introduction of the new diet). Rats were anesthetized with sodium pentobarbital, and blood was obtained both from the portal vein and from the heart with heparinized syringes. Blood was immediately centrifuged, and plasma was collected and frozen. Liver, kidneys and brain were removed and immediately frozen in liquid nitrogen for subsequent measurement of cysteamine, hypotaurine and taurine levels and for assay of cysteamine dioxygenase activity.

2.4. Measurement of Cysteamine, Hypotaurine and Taurine by High Performance Liquid Chromatography

2.4.1. Standards and Sample Preparation

Stock standard solutions of cysteamine, hypotaurine, and taurine (Sigma Chemical Co., St.Louis, MO) were prepared in deionized water and stored at -20^{o}C until used. Liver, brain, kidney and plasma samples were obtained from mice and rats as described below and stored at -80^{o}C until used. Frozen tissues from *Vanin 1* -/- and +/+ mice were obtained from Dr. Franck Galland (Centre d'Immunologie de Marseille-Luminy, INSERM-CNRS-Universite de la Mediterranee, France). Frozen tissues were homogenized in ice-cold 5% (w/v) sulfosalicylic acid to prepare 20% (w/v) homogenates. Homogenates were then centrifuged at 10,000xg for 10 min to obtain the acid supernatants. One volume of plasma was mixed with four volumes of 5% sulfosalicylic acid. Acid supernatants from cysteamine dioxygenase assay mixtures were prepared as described above.

2.4.2. Sample Derivatization and HPLC Measurement of Cysteamine, Hypotaurine and Taurine

To 0.5 ml of acid supernatant was added 50 µl of m-cresol purple (0.2 mM) with mixing. While mixing the sample in a vortex mixer, 0.48 ml of 2 M KOH/ 2.4 M $KHCO_3$ was added, followed by the addition of 50 µl of 50 mM dithiothreitol (DTT). The mixture was incubated at 37^{o}C for 30 min. After incubation, 50 µl of 200 mM iodoacetate was added with mixing, and the mixture was allowed to stand in the dark for 10 min to alkylate the thiol groups.

Chromatography of derivatized samples was carried out on a 4.6 x 150 mm column packed with Nova-Pak C_{18} packing materials (4 μm spherical particles) (Waters Corp., Milford, MA) equipped with a guard C_{18} cartridge (5 μm spherical particles) (Alltech Associates, Inc., Deerfield, IL). Under conditions of no-flow, 75 μl of o-phthalaldehyde (OPA)-2-mercaptoethanol derivatizing reagent and then a 50 μl volume of the sample acid supernatant or standard solution were injected into the pre-column tubing by an automatic sample injector (WISP Model 712, Waters Corp., Milford, MA). The derivatizing reagent was prepared fresh daily by mixing 3.5 mg OPA with 50 μl 95% (v/v) ethanol, 5 ml 100 mM borate buffer (pH 10.4), and 10 μl of 2-mercaptoethanol. A 3-minute delay was programmed prior to initiating flow of mobile phase to allow reaction of amines with OPA.

A gradient mobile phase was used. Buffer A was 100 mM potassium phosphate buffer-3% (v/v) tetrahydrofuran (THF), pH 7.0, and buffer B was 100 mM potassium phosphate buffer-3% THF-40% (v/v) acetonitrile, pH 7.0. The buffers were filtered through a 0.45 μm filter (Micron Separations, Inc., Westboro, MA) before use. The flow rate of the mobile phase was 1.0 ml/min. The column was at room temperature. The mobile phase was started isocratically for the first 1.0 min at 3% buffer B, increased to 30% buffer B over 6 min, to 55% buffer B over 13 min, and then to 100% buffer B over 2 min. At 22 min into the run, the mobile phase was decreased to 3% buffer B over 10 min and the column was allowed to equilibrate for another 11 min before the next sample was injected. Detection of amino acids was done using a Spectra/glo Filter Fluorometer (Gilson Medical Electronics, Middleton, WI) equipped with a 5 μl flow cell and filters for excitation and emission peaks at 360 and 455 nm, respectively. The fluorometer was connected to a personal computer equipped with Peak Simple Chromatography Data System version 3.21 (LabAlliance, State College, PA) for integration of chromatographic peaks.

3. RESULTS

3.1. Measurement of Cysteamine, Hypotaurine and Taurine

The HPLC procedure allowed measurement of cysteamine, hypotaurine and taurine in the same run. It was necessary to block the sulfhydryl group of cysteamine prior to OPA derivatization. A sample chromatogram for standards is shown in Fig. 2. This method was very sensitive, allowing detection of picomolar amounts of cysteamine and hypotaurine.

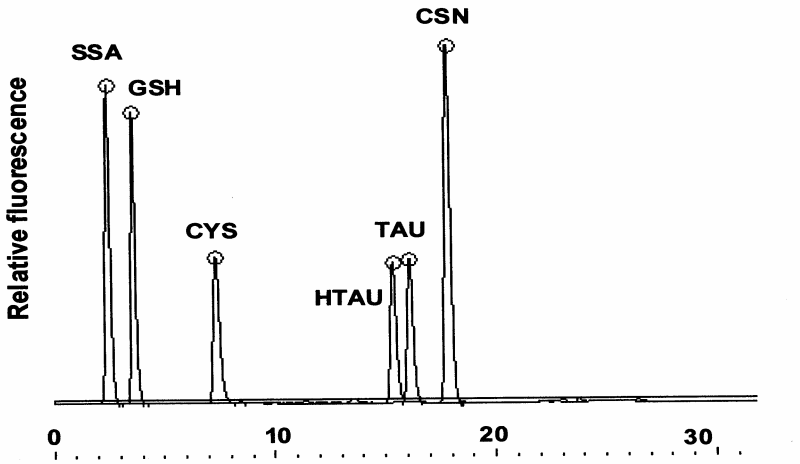

Figure 2. Chromatogram of HPLC separation of mixture of standards: 100 μM each of glutathione (GSH), cysteine (CYS), hypotaurine (HTAU) and taurine (TAU) and 250 μM of cysteamine (CSN). SSA = sulfosalicylic acid.

3.2. Cysteamine Dioxygenase Activity in Rat and Mouse Tissues

The rate of conversion of cysteamine to hypotaurine + taurine was assayed in mouse and rat tissues using assay conditions based on those reported by Cavallini *et al.* (1966). The assay system contained 3.3 mM cysteamine and was buffered at pH 7.6. Although sulfide was included in the assays used by Cavallini *et al.* (1966), we found no effect of sulfide addition on the rate of conversion of cysteamine to hypotaurine. Hypotaurine and taurine were measured by HPLC; no increases in taurine were observed so activity was calculated solely based on the increase in hypotaurine concentration.

Activity in mouse liver was 91 nmol hypotaurine produced per min per g tissue; activity in mouse kidney was 37 nmol hypotaurine produced per min per g tissue. Activity was lower in rat tissues: 26 nmol hypotaurine per min per g tissue for liver and 21 nmol hypotaurine per min per g tissue for kidney.

3.3. Short-Term Exposure to High Protein or High Cysteamine Does Not Affect Tissue Cysteamine Dioxygenase Activity in Rat Tissues

Rats switched from a high protein diet to a low protein diet or to a low protein diet supplemented with cysteamine did not demonstrate a change in tissue cysteamine dioxygenase activity over the course of 10 h. Hepatic activities are reported in Table 1. The activity of cysteamine dioxygenase in kidney averaged 3.5 ± 0.4, and that in brain was 3.7 ± 0.5 nmol hypotaurine per h per mg protein. Renal and brain enzyme activity was not affected by short-term exposure to the low protein or the cysteamine-supplemented diet. The possible effect of a long-term change of diet was not investigated.

Table 1. Cysteamine dioxygenase activity in liver of rats fed various diets

	Cysteamine dioxygenase activity nmol hypotaurine per h per mg protein	
Diet	6 h	10 h
Low Protein	4.5 ± 0.3	3.9 ± 0.2
Low Protein + Cysteamine	4.3 ± 0.3	4.1 ± 0.4
High Protein	4.4 ± 0.7	3.7 ± 0.4

Values are means ± SD for 3 rats.

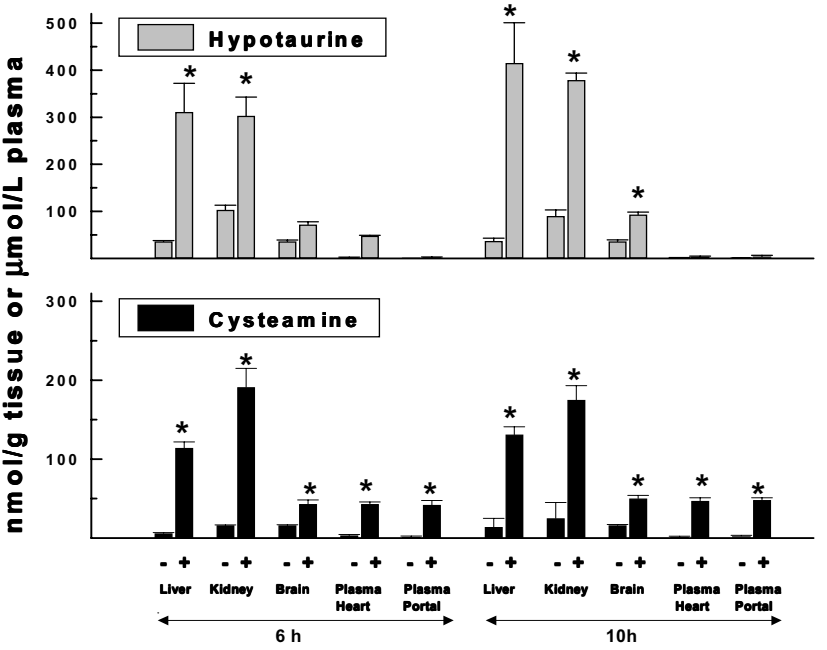

Figure 3. Hypotaurine and cysteamine levels in tissues and plasma of rats fed either a low protein diet (100 g casein/kg diet) or a cysteamine-supplemented low protein diet (100 g casein + 7.2 g cysteamine/kg diet) for 6 or 10 hours. Prior to introduction of the experimental diet, rats were adapted to a high-protein diet with food available only during the dark period. Following the normal 12-h fasting/light period, rats were given either the low protein or low protein + cysteamine diet and then killed 6 or 10 hours later. An asterisk (*) indicates that the value for the cysteamine-supplemented group (+) is significantly different than that for the low-protein fed group (-) at $P \leq 0.05$ by ANOVA and Tukey's procedure. Values are means ± SD for 3 rats.

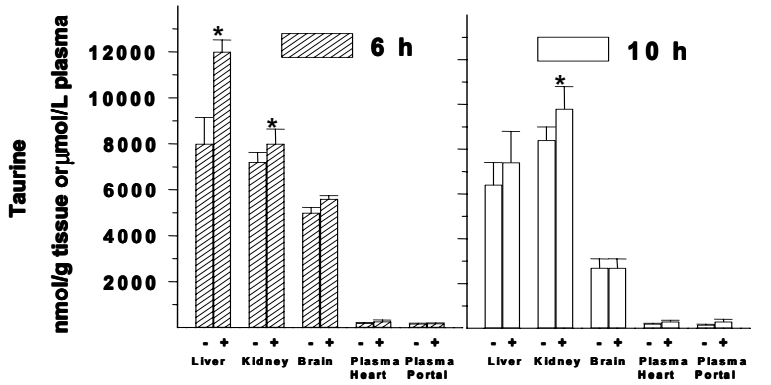

Figure 4. Taurine level in tissues and plasma of rats fed either a low protein diet (100 g casein/kg diet) or a cysteamine-supplemented low protein diet (100 g casein + 7.2 g cysteamine/kg diet) for 6 or 10 hours. An asterisk (*) indicates that the value for the cysteamine-supplemented group (+) is significantly different than that for the low-protein fed group (-) at $P \leq 0.05$ by ANOVA and Tukey's procedure. Values are means ± SD for 3 rats.

3.4. Rats Fed Cysteamine Have Elevated Levels of Hypotaurine and Taurine in Tissues

Rats that had been adapted to a high protein diet (400 g casein/kg diet), with food available only during the dark cycle, were introduced to diets that contained 100 g casein with or without 7.2 g cysteamine per kg diet at the beginning of the dark cycle and then killed after 6 or 10 hours. Cysteamine and hypotaurine levels in these rats are shown in Fig. 3. Cysteamine levels in plasma, liver, kidney and brain of rats fed the cysteamine-supplemented diet were significantly and substantially higher than those in tissues of rats fed the basal diet. Rats fed the cysteamine-supplemented diet had markedly elevated levels of hypotaurine in liver, kidney and brain, but not in plasma. Taurine levels were also substantially elevated in liver of rats at 6 hours and in kidney of rats at both 6 and 10 hours, as shown in Fig. 4.

For comparison, we looked at the hypotaurine level in tissues of rats fed an equimolar amount of supplemental cysteine. As shown in Fig. 5, tissue and plasma hypotaurine levels were higher in rats fed a diet supplemented with cysteine than in those fed a diet supplemented with an equimolar amount of cysteamine. This is consistent with cysteine being more readily converted to hypotaurine as a result of cysteine dioxygenase and cysteinesulfinate decarboxylase activities. Nevertheless, the increase in hypotaurine level in liver and kidney of rats given supplemental cysteamine was 42% and 52% as much (at 6 hours), respectively, of that observed in rats given an equimolar amount of supplemental cysteine, demonstrating that cysteamine is a good precursor of hypotaurine *in vivo*. Supplementation of the diet with cysteine had no effect on tissue cysteamine concentrations, and supplementation of the diet with cysteamine had no effect on tissue cysteine concentrations.

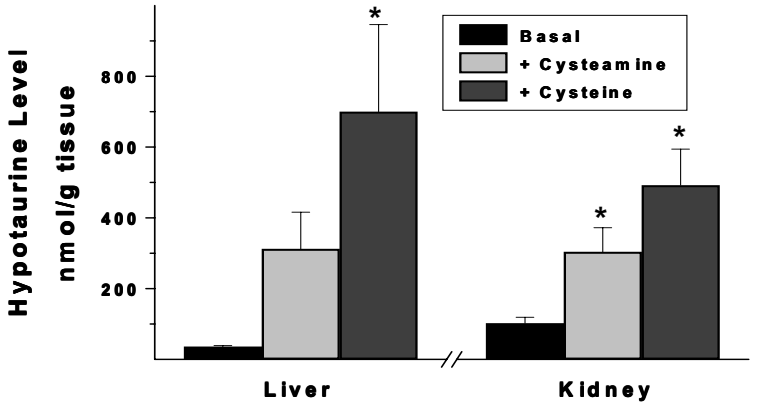

Figure 5. Hypotaurine level in liver and kidney of rats fed a low protein diet (100 g casein/kg diet), a cysteamine-supplemented low protein diet (100 g casein + 7.2 g cysteamine/kg diet) or a cysteine-supplemented low protein diet (100 g casein + 8.1 g cysteine/kg diet) for 6 hours. An asterisk (*) indicates that the value for the cysteamine- or cysteine-supplemented group (+) is significantly different than that for the low-protein fed group (-) at $P \leq 0.05$ by ANOVA and Tukey's procedure. Values are means ± SD for 3 rats.

3.5. Hypotaurine Is Not Elevated in Tissues of *Vanin-1* Knockout Mice

Pitari *et al.* (2000) reported that cysteamine levels were 15 nmol/g [~75 pmol/mg protein] for kidney and 24 nmol/g [~120 pmol/mg protein] for liver of wild-type mice but undetectable in tissues of *Vanin-1* knockout mice. Based on the reported lack of membrane-bound pantetheinase activity in the *Vanin-1* knockout mouse that resulted in very low cysteamine levels in knockout mice compared to their wild-type littermates, we hypothesized that *Vanin-1* knockout mice would also have lower hypotaurine levels than wild-type mice. However, we did not observe lower hypotaurine levels in *Vanin-1 (-/-)* mice. Hypotaurine was not detected in liver or brain of either *Vanin-1 (+/+) or Vanin-1 (-/-)* mice that had been fed a non-purified rodent diet. Hypotaurine was measurable in kidney, but levels were significantly higher, rather than lower, in kidneys of the knock-out mice compared to wild-type mice.

It is also worth noting that we did not observe large differences in tissue cysteamine and glutathione (GSH) levels as compared to the much lower cysteamine and higher GSH levels that have been reported by other investigators for the *Vanin-1* knockout mouse (Pitari *et al.,* 2000; Berruyer *et al*., 2004).

Table 2. Glutathione, cysteine, taurine, hypotaurine and cysteamine levels in liver, kidney and brain of *Vanin-1 -/-* and *Vanin-1 +/+* mice

	Wild-type mice	*Vanin-1* knockout mice
LIVER	μmoles per g tissue	
GSH (total, after reduction)	7.5 ± 0.3	6.8 ± 1.1
Cysteine (total, after reduction)	0.088 ± 0.004	0.088 ± 0.007
Taurine	1.9 ± 0.5	2.5 ± 1.0
Hypotaurine	ND	ND
Cysteamine	0.16 ± 0.01	0.09 ± 0.08
KIDNEY		
GSH (total, after reduction)	2.6 ± 0.9	2.6 ± 0.3
Cysteine (total, after reduction)	0.75 ± 0.25	0.58 ± 0.05
Taurine	2.2 ± 0.3	2.4 ± 0.4
Hypotaurine	0.06 ± 0.01	0.13 ± 0.01*
Cysteamine	0.35 ± 0.14	0.23 ± 0.02
BRAIN		
GSH (total, after reduction)	2.0 ± 0.2	2.0 ± 0.2
Cysteine (total, after reduction)	0.13 ± 0.01	0.09 ± 0.02
Taurine	3.0 ± 0.1	2.7 ± 0.3
Hypotaurine	ND	ND
Cysteamine	0.11 ± 0.10	ND

*Significantly different from wild-type value at $P \leq 0.05$. ND = not detected.

4. DISCUSSION

These results collectively support a role of the coenzyme A → cysteamine → hypotaurine metabolic pathway in mammalian tissues. Significant cysteamine dioxygenase activity was observed in rat and mouse tissues, particularly if considered in light of the relatively low levels of cysteine dioxygenase activity present in tissues of animals fed low protein diets (Stipanuk *et al*., 2002). However, the cysteamine dioxygenase activities we report here are substantially lower than those reported by Cavallini and coworkers (Duprè and DeMarco, 1964; Federici *et al*., 1980). This may be due to improved methodology resulting in more accurate and precise measurements of hypotaurine production. The earlier assays made use of radiolabeled cysteamine as substrate and involved quantitation of radiolabeled product by paper chromatography or measurement oxygen consumption (Duprè and DeMarco, 1964; Federici *et al*., 1980).

Hypotaurine was detected in tissues of rats during the 10-h period of low protein-diet consumption. Hypotaurine averaged 35 nmol/g in the liver, 95 nmol/g in the kidney, 25 nmol/g in the brain, and 1 μmol/l in arterial plasma. These levels were higher in rats given the diet containing cysteamine: 360 nmol/g in liver, 340 nmol/g in kidney, 60 nmol/g in brain, and 4 μmol/l in arterial plasma. Even higher levels of hypotaurine were observed in tissues of rats fed a diet containing excess cysteine. Assessment of tissue hypotaurine levels in rats fed cysteamine vs cysteine in the diet clearly indicates that cysteamine can be converted to hypotaurine at a physiologically significant rate. Depending upon the rate of cysteamine production via coenzyme A turnover, cysteamine could be a quantitatively important precursor of taurine.

The levels of hypotaurine reported herein are consistent with several other recent reports of hypotaurine levels in tissues of rats fed diets with varying sulfur amino acid content. Kasai *et al.* (1992) fed rats a diet with 80 g casein/kg (*vs*. 100 g/kg in our low protein diet) without or with 30 g methionine/kg (*vs*. 7.2 g cysteamine or 8.1 g cysteine/kg in our supplemented treatment diets). They reported hypotaurine levels of 100 nmol/g in muscle, 440 nmol/g in kidney, and 200 nmol/g in spleen of rats fed the low casein diet. These levels are higher than those we observed in rats fed a slightly higher amount of casein in the diet; this may relate to net tissue breakdown in the rats fed the diet with only 80 g casein/kg as these animals were not growing. Hypotaurine levels were increased to 1030 nmol/g in muscle, 1400 nmol/g in kidney, and 800 nmol/g in spleen of rats fed the diet with excess methionine for 7 or 14 days. Hypotaurine is likely excreted in the urine, along with taurine, under conditions of very high sulfur amino acid intake, based on excretion of hypotaurine by rats given hypotaurine by intraperitoneal injection (Fujiwara *et al*., 1995). Although taurine levels consistently increase by a greater absolute amount than do hypotaurine levels, it is nevertheless evident that the capacity for oxidation of hypotaurine to taurine is exceeded at high rates of hypotaurine production, resulting in the accumulation of hypotaurine in tissues.

The *Vanin-1* knockout mouse initially appeared to be a useful model for the study of coenzyme A turnover because of the reported lack of pantetheinase and accumulation of cysteamine. Our observations, however, suggest that the block in pantetheine hydrolysis is not complete in the *Vanin-1* knockout model. It is known that mice express at least two *Vanin* genes, *Vanin-1* and *Vanin-3* (Martin *et al*., 2001), so it is possible that other pantetheinases compensate for the lack of Vanin-1. Our data also indicate that tissue GSH levels are similar in *Vanin-1* knockout and wild-type mice, indicating that it is very unlikely that changes in GSH levels, as has been suggested, are responsible for the increased resistance to oxidative injury that has been observed in *Vanin-1* knockout mice (Berruyer *et al*., 2004).

5. ACKNOWLEDGMENTS

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IN VIVO REGULATION OF CYSTEINE DIOXYGENASE VIA THE UBIQUITIN-26S PROTEASOME SYSTEM

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1. INTRODUCTION

The intracellular free amino acid pool of cysteine is tightly regulated in the mammalian liver. In rats, for instance, intracellular cysteine is maintained between 20 and 100 μmol/g even when dietary protein or sulfur amino acid intake is varied from sub-requirement to above-requirement levels for this species (Lee *et al.*, 2004). The narrow range of permissible cysteine concentrations is the consequence of two homeostatic requirements. Liver tissue must keep cysteine levels sufficiently high to meet the needs of protein synthesis and the production of other essential molecules like glutathione, coenzyme A, taurine, and inorganic sulfur. At the same time, however, cysteine concentrations must also be kept below the threshold of cytotoxicity.

An important enzyme that contributes to the regulation of steady-state intracellular cysteine levels is cysteine dioxygenase (CDO, EC 1.13.11.20). Expressed at high levels in the liver with lower levels in the kidney, brain, and lung, this Fe^{2+} metalloenzyme catalyzes the addition of molecular oxygen to the sulfhydryl group of cysteine, yielding cysteinesulfinate. The oxidative catabolism of cysteine to cysteinesulfinate represents an irreversible loss of cysteine from the free amino acid pool; cysteinesulfinate is shuttled into numerous metabolic pathways including hypotaurine/taurine synthesis, inorganic sulfur production, and use of the carbon backbone as pyruvate for gluconeogenesis or oxidative decarboxylation and cellular respiration. *In vivo* data suggest that the liver, the organ with the highest amount of CDO expression, uses CDO as a means of disposing excess cysteine obtained through the diet as well as to provide the essential metabolites sulfate, hypotaurine, and taurine (Garcia and Stipanuk, 1992).

Steady-state levels of hepatic CDO protein are exquisitely regulated by dietary sulfur amino acids. Hepatic CDO activity is barely detectable in rats fed low-protein (i.e.,

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sulfur amino acid poor) diets, but increases as much as 35-fold in rats fed diets enriched with methionine, cystine, or total protein (Bella *et al.*, 1999a,b). The regulation of CDO appears to be specifically associated with changes in intracellular cysteine concentration; other non-sulfur amino acids have no effect on CDO levels (Kwon *et al.,* 2001). Cysteine's ability to regulate CDO levels is rather unique in that it is an exclusively post-translational phenomenon (Bella *et al.*, 2000). As demonstrated in rat primary hepatocyte cultures, high levels of cysteine significantly prolong the half-life of CDO by decreasing its ubiquitination and subsequent degradation via the 26S proteasome system (Stipanuk *et al.*, 2004).

Because previous work describing the cysteine-dependent regulation of CDO by the ubiquitin-26S proteasome system has been limited to cell culture models, we decided to explore whether this same system is responsible for the regulation of hepatic and kidney CDO protein *in vivo*. We accomplished this by pharmacological inhibition of the 26S proteasome complex. We also evaluated whether this inhibition had any effect on hypotaurine/taurine metabolism as would be predicted by a perturbation in steady-state CDO levels.

2. MATERIALS AND METHODS

2.1. Animal Feeding Studies

Male Sprague-Dawley rats (170-210 g) were purchased from Harlan Sprague Dawley (Indianapolis, IN). Rats were housed in polycarbonate cages containing paper bedding in a room maintained at 20°C and 60-70% humidity with light from 18:00 h to 06:00 h. These animals had *ad libitum* access to water but had access to food only during the dark cycle 06:00 h to 18:00 h, wherein food was provided in ceramic cups. To ensure high initial levels of hepatic CDO protein, all rats were fed a high protein diet (HP) for one week prior to the treatment day. This diet, prepared by Dyets, Inc. (Bethlehem, PA), contained 40% casein by weight. On the treatment day, rats were randomly assigned to the following experimental groups: maintenance on high protein (HP) diet, switch to a low protein (LP, 10% casein by weight) diet, switch to a low protein diet supplemented with 8.12 g/kg diet cysteine (LP+CYS), or switch to a low protein diet (LP+PII) plus an intraperitoneal injection of the specific proteasome inhibitor, proteasome inhibitor I (PII, 17 mg/kg in DMSO). The LP diet mix, made by Dyets, Inc., was prepared with 25 g/kg of sucrose excluded from it. For the LP and LP+PII diets, all of this sucrose was added back in. For the LP+CYS diet, 16.88 g/kg sucrose was added along with 8.12 g/kg cysteine to fully reconstitute the diet. All diets were prepared as gel cubes by the addition of a hot 3% (w/v) agar solution, followed by casting in containers at 4°C and cutting into easily managed cubes.

At the end of the fasting light cycle on treatment day (time = 0 h), 3 rats were killed to establish baseline values and the remaining rats were switched to their assigned dietary treatments (6 rats/treatment). Animals were subsequently killed 6 h and 10 h after the diet switch (3 rats from each group per time point). Animals in the LP+PII group received an IP injection of PII 2.5 h after the diet switch. At the appropriate time point, rats were weighed, and anesthetized using sodium pentobarbital (30 mg/ml in 15% v/v ethanol) at a dose of 90 mg/kg. Ventricular blood was collected into heparinized (180 U/syringe) syringes by cardiac puncture, transferred to microfuge tubes, and centrifuged

for 3 minutes at 3,000xg. The plasma was removed and frozen in liquid nitrogen. Whole livers and kidneys were removed, rinsed with ice-cold saline, and immediately frozen in liquid nitrogen. The experimental protocol used in this study was approved by the Cornell University Institutional Animal Care and Use Committee.

2.2. Western Blot Analysis

Western blot analysis was conducted as previously described but with some minor modifications (Bella *et al.,* 1999b). Briefly, livers were homogenized in a lysis buffer (20% w/v) containing 50 mM Tris-Cl, 150 mM NaCl, 1 mM EDTA, 0.5% v/v NP-40, 10 mM ortho-vanadate, 1x protease inhibitor cocktail (Sigma, St. Louis, MO), 10 mM N-ethylmaleimide, and 20 μM MG-132 (Boston Biochem, Boston, MA), pH=7.4. Homogenates were centrifuged at 16,500xg for 20 min. Supernatant proteins were separated by one-dimensional SDS-PAGE (either 12% or 15% w/v acrylamide) and then electroblotted overnight onto 0.45 μm Immobilin-P PVDF membranes (Millipore Corporation, Medford, MA). Immunoreactive protein was detected by chemiluminescence using rabbit anti-rat CDO polyclonal antibody (Stipanuk *et al.,* 2004a) and HRPO-conjugated goat anti-rabbit secondary antibody (Supersignal Pico, Pierce) with exposure to Kodak X-OMAT film. Developed films were scanned using a desktop scanner. With the obtained electronic images, two-dimensional quantitative densitometric analysis was performed on areas of interest using AlphaEase software (Alpha Innotech, San Leandro, CA). The apparent molecular weights of native CDO (which runs as a double band on SDS-PAGE with a molecular weight of ~23 kDa) and ubiquitinated CDO (~23 kDa + n•8 kDa, where n = the number of attached ubiquitin moieties) were consistent with previously published values (Yamaguchi *et al.*, 1978; Stipanuk *et al.,* 2004b).

2.3. Analysis of Tissue Hypotaurine and Taurine Content by High Performance Liquid Chromatography (HPLC)

Acid extracts of tissue homogenates were prepared by homogenizing frozen liver samples in 4 volumes of 5% (w/v) sulfosalicylic acid (SSA). Homogenates were then centrifuged at 10,000xg for 10 min. One-half milliliter of acid supernatant was removed and added to 50 μl of 0.2 mM m-cresol purple with mixing. While mixing the sample, 0.48 ml of 2 M KOH/2.4 M $KHCO_3$ and subsequently 50 μl of 50 mM dithiothreitol were added. The mixture was incubated at 37°C for 30 min. After the incubation, 50 μl of 200 mM iodoacetate was added with mixing, and the mixture was placed in the dark for 10 min to alkylate free thiols.

Chromatography of derivatized samples was conducted on a 4.6 x 150 mm column packed with Nova-Pak C_{18} 4 μm spherical packing material (Waters Corp., Milford, MA) equipped with a C_{18} guard cartridge (5 μm spherical particles; Alltech Associates, Inc., Deerfield, IL). Samples and standards were derivatized with o-phthalaldehyde (OPA) prior to injection onto the column using an automatic sample injector (WISP Model 712, Waters Corp., Milford, MA). Under conditions of no-flow, 75 μl of OPA reagent was injected, and this was followed by injection of 50 μl of the standard or sample solution. The OPA reagent was made fresh each day by mixing 3.5 mg OPA with 50 μl 95% ethanol, 5 ml 100 mM borate buffer (pH = 10.4), and 10 μl 2-mercaptoethanol. A

programmed 3-minute delay before the initiation of flow allowed sufficient time for amines to react with the OPA.

Amino acids were separated by gradient elution using two buffers. Buffer A was 100 mM potassium phosphate buffer plus 3% (v/v) tetrahydrofuran (THF), pH=7.0, and buffer B was 100 mM potassium phosphate buffer plus 3% (v/v) tetrahydrofuran (THF) and 40% (v/v) acetonitrile, pH=7.0. Buffers were filtered through 0.45 μm filters before use. Flow rate was 1.0 ml/min and column temperatures were maintained at room temperature. Mobile phase was started isocratically for the first minute at 3% B and then gradients were run by increasing to 30% B over 6 min and to 55% buffer B over 13 min. The column was washed by increasing Buffer B to 100% over 2 min and holding at 100% B for 3 minutes. At 25 min into the run, the mobile phase was decreased to 3% B over 10 min and the column was allowed to equilibrate for another 11 min before the next sample injection.

Detection of OPA-derivatized amino acids was done using a Spectra/glo Filter Fluorometer (Gilson Medical Electronics, Middleton, WI) equipped with a 5 μl flow cell and filters for excitation and emission peaks at 360 and 455 nm, respectively. The fluorometer was connected to a personal computer equipped with Peak Simple Chromatography Data System version 3.21 (LabAlliance, State College, PA) for the integration of chromatographic peaks.

2.4. Statistics

All quantitative data are expressed as means ± standard deviations. Statistical analyses were conducted by ANOVA and Tukey's post-test procedure using Prism 3 (GraphPad Software, Inc., San Diego, CA). Differences were considered significant at $P \leq 0.05$. Unless otherwise stated, fold changes are relative to 0 h values.

3. RESULTS

Fig. 1 shows the effect of each treatment on the expression of hepatic CDO protein. Rats switched from the HP diet to the LP diet exhibited a significant decrease in CDO protein over the time course of the study; by the 10 h time point, CDO levels had fallen by more than 80%. CDO levels were stabilized, however, by maintaining animals on the HP diet, supplementing the LP diet with cysteine, or providing an injection of PII. Although the stabilization of CDO by PII was comparable to that of rats maintained on the HP diet or given the LP+CYS diet when measured at 6 h, the ability of PII to attenuate CDO degradation was diminished by approximately 50% between 6 and 10 h. Nevertheless, animals fed the LP diet and receiving a PII injection retained significantly more CDO protein than did animals fed the LP diet alone.

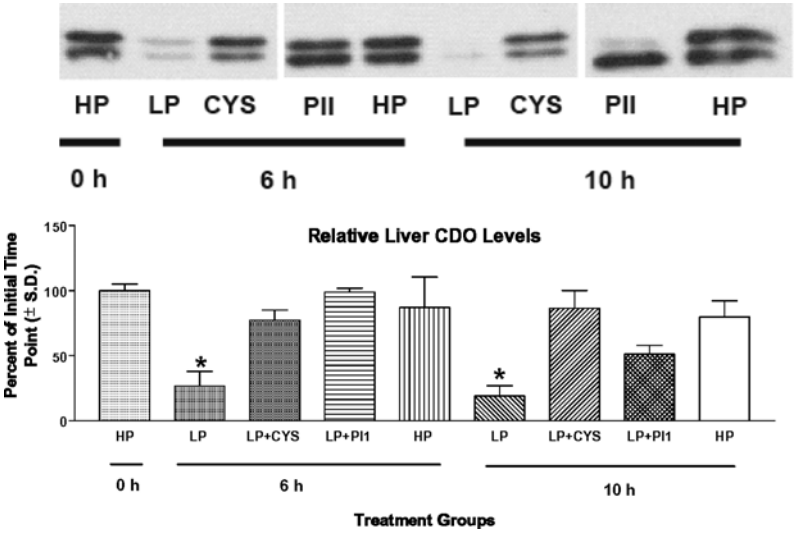

Figure 1. The effects of dietary manipulation and proteasome inhibition on levels of liver CDO protein in rats. For SDS-PAGE analysis of CDO, 60 μg of soluble liver protein was loaded per lane on a 12% polyacrylamide gel. A representative Western is shown for one such analysis (*Top*). To determine the relative changes in protein levels for all samples, the optical density of each CDO band was measured by densitometry. Optical densities were then normalized by HP 0 h control values (run on each gel) and expressed as a percent of these controls in the bar graph (*Bottom*). HP, high protein diet; LP, low protein diet; LP+CYS, low protein diet + 8.12 g cysteine/kg diet; LP+PII, low protein diet + IP injection of proteasome inhibitor I (17 mg/kg). * $P<0.01$ *vs*. 0 h HP group.

In the kidney, there was a slight decrease in the expression of CDO following a switch to the LP diet (Fig. 2). This effect was not significant. Supplementing the LP diet with cysteine produced a significant 2.5-fold increase in kidney CDO levels that was maintained up to at least 10 h after the diet switch. Proteasome inhibitor I also increased kidney CDO levels by ~2.0-fold over the course of the experiment, though due to sample variability this effect was not significantly different from either the LP or LP+CYS group.

Primary hepatocyte culture studies have shown that a reduction in cysteine availability causes an increase in the steady state levels of ubiquitinated CDO (Stipanuk *et al.,* 2004a). These ubiquitinated species, which constitute a relatively small fraction of the total CDO protein pool, are seen as a higher molecular weight ladder of bands on Western blots. The ubiquitinated CDO migrated with apparent molecular weights that differed from that of CDO by integer multiples of approximately 8 kDa. As seen in Fig. 3, mono- and di-ubiquitinated forms of CDO were present in the livers of rats regardless of the treatment group. There was a marked accumulation of these species in the LP+PII-treated animals, particularly at the 10 h time point, as would be expected from conditions wherein CDO ubiquitination is enhanced and proteasome activity is inhibited. Higher molecular weight forms of ubiquitinated CDO are also apparent at the 10 h time point, further demonstrating the accumulation of modified CDO following proteasome inhibition.

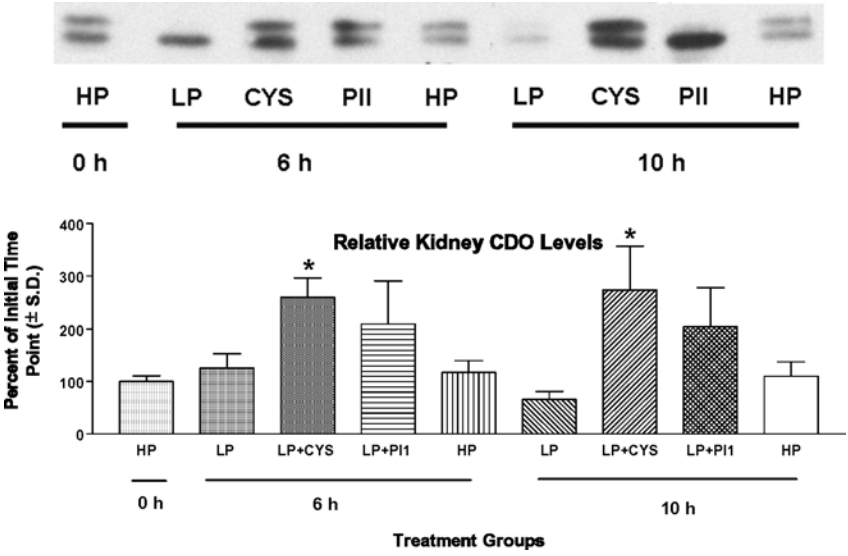

Figure 2. The effects of dietary manipulation and proteasome inhibition on levels of kidney CDO protein in rats. For SDS-PAGE analysis of CDO, 100 μg of soluble kidney protein was loaded per lane on a 12% polyacrylamide gel. A representative Western is shown for one such analysis (*Top*). To determine the relative changes in protein levels for all samples, the optical density of each CDO band was measured by densitometry. Optical densities were then normalized by HP 0 h control values (ran on each gel) and expressed as a percent of these controls in the bar graph (Bottom). * $P<0.05$ *vs*. the initial (0 h HP) value.

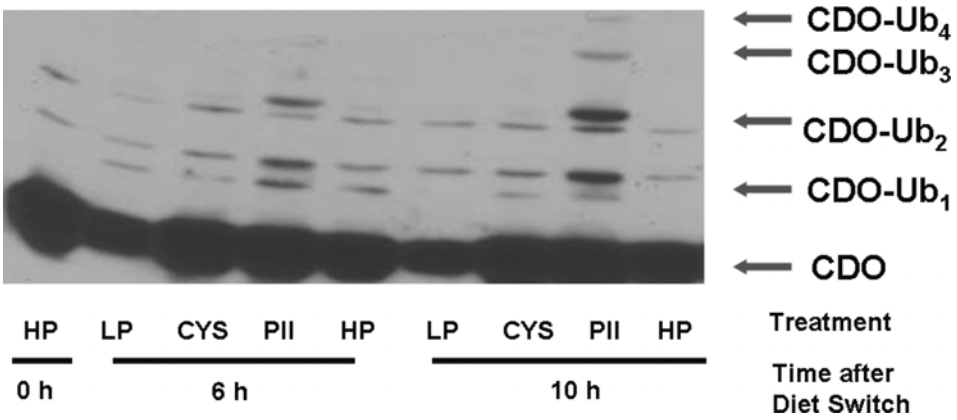

Figure 3. A Western blot showing the effects of dietary manipulation and proteasome inhibition on steady state levels of ubiquitinated liver CDO. 125 μg of liver homogenate was loaded into each lane. Ubiquitinated species are designated by CDO-Ub, with the total number of attached ubiquitin moieties indicated by a numeral subscript.

Normalization of the amount of ubiquitinated CDO by the relative quantity of unmodified CDO protein present in each sample enables a clearer depiction of the treatment-specific effects on steady state ubiquitination (Fig. 4). In the LP+CYS and HP groups, the relative amount of ubiquitinated CDO did not change significantly. Switching to the LP diet, by contrast, produced a significant increase in the relative amount of ubiquitinated CDO: 3.7-fold after 6 h and 5.9-fold after 10 h. At 6 h, 4.5 h after the PII injection, there was no enhancement of the relative amount of ubiquitinated CDO by PII above that achieved by the LP diet alone. By 10 h, however, there was a significant 9.3-fold increase in the relative amount of accumulated ubiquitinated CDO.

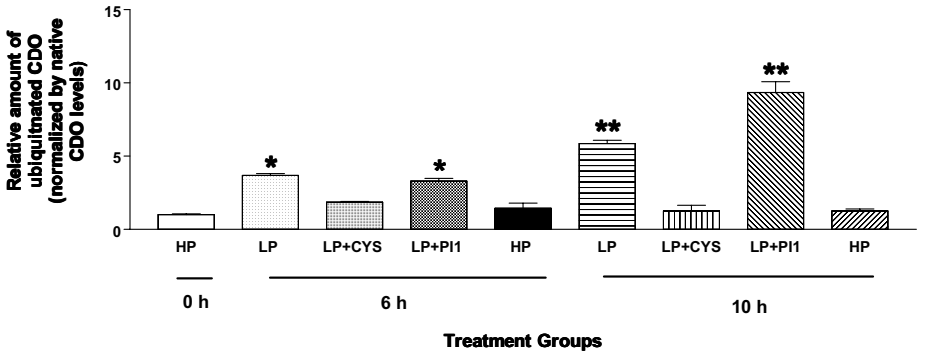

Figure 4. Changes in ubiquitinated hepatic CDO levels, normalized by the relative amount of unmodified (native) CDO in each sample. Normalization of ubiquitinated CDO by the relative amount of CDO provides a clearer representation of ubiquitination kinetics. This is due to the fact that the total quantity of ubiquitinated protein, the product of a first-order reaction, will be related in part to the amount of unmodified protein available for ubiquitination. Therefore, to determine changes in the steady-state kinetics of ubiquitination under conditions in which the pool of unmodified target protein is changing, normalization by the unmodified protein pool is needed. * $P<0.01$ *vs*. initial (0 h HP) value; * $P<0.001$ *vs*. initial (0 h HP) value.

Given the effectiveness of proteasome inhibition in stabilizing CDO levels *in vivo*, we asked whether there was an accompanying alteration in the downstream synthesis of hypotaurine and taurine. In the pathway leading to hypotaurine/taurine synthesis from cysteine, CDO catalyzes the first step by producing cysteine sulfinate. Cysteinesulfinate is then decarboxylated by cysteinesulfinate decarboxylase (CSD) to yield hypotaurine. Hypotaurine is finally oxidized to taurine by a process that has yet to be understood. The principal regulator of flux through this pathway has been alternately ascribed to either CDO or CSD, depending upon the model organism employed in the study (De la Rosa *et al.,* 1985, 1987; Drake *et al.*, 1987). However in the rat it is clear that, while intracellular cysteinesulfinate levels are very low (frequently beneath the limits of detection), the decarboxylation of cysteine sulfinate does not appear to be the rate limiting step in the overall flux of cysteine to hypotaurine/taurine (i.e., the flux of cysteine to hypotaurine/taurine is more affected by synthesis of cysteinesulfinate by cysteine dioxygenase than it is by changes in cysteinesulfinate decarboxylase activity *in vivo*) (Drake *et al.,* 1987). Thus, the production of cysteinesulfinate by CDO appears to be the rate-limiting step in the generation of hypotaurine/taurine under physiological conditions (Stipanuk, 2004).

Concordant with the idea that CDO is an important enzyme in the control of hypotaurine synthesis, there was a clear association between organ CDO protein levels and organ hypotaurine content in this study. In the liver, decrements in CDO protein within the LP group were associated with a sharp decline in hypotaurine content (Table 1). The LP+CYS, LP+PII, and HP groups, which had elevated levels of CDO protein relative to the LP groups, displayed elevated levels of liver hypotaurine. A similar trend was observed in the kidney. LP+CYS and LP+PII rats, which expressed higher levels of renal CDO than the LP or HP groups, tended to accumulate larger amounts of hypotaurine than the other dietary treatment groups. In the plasma compartment, hypotaurine values followed a slightly different pattern from those seen for the liver and kidney. Plasma hypotaurine levels were not elevated in rats continued on the HP diet but were elevated in rats switched to the LP+CYS diet. Elevated plasma hypotaurine was observed for rats in the LP+PII treatment group at 6 h, but hypotaurine had returned to initial (0 h HP) levels by 10 h.

Table 1. Effects of experimental diets and proteasome inhibition on hypotaurine levels in liver, kidney, and plasma

Time (h)	0	6			
	[Fasting]	*[Fed]*			
Diet	*HP*	*LP*	*LP+CYS*	*LP+PII*	*HP*
Liver	158±25[a]	37±3[b]	701±247[c]	685±123[c]	405±121[c]
Kidney	179±27[a]	102±17[a]	492±102[b]	432±17[b]	166±16[a]
Plasma	0.5±0.2[a]	0.4±0.2[a]	2.7±0.9[b]	2.8±0.6[b]	0.4±0.2[a]
Time *(h)*		10			
		[Fed]			
Diet		*LP*	*LP+CYS*	*LP+PII*	*HP*
Liver		37±10[b]	999±516[c]	249±191[ac]	337±75[ac]
Kidney		91±24[a]	685±218[b]	315±147[ab]	183±29[a]
Plasma		0.2±0.2[a]	3.5±1.4[b]	0.3±0.4[a]	0.2±0.2[a]

The unit for liver and kidney values is pmol/mg protein. The unit for plasma data is µmol/dl. Within a given tissue, values with different superscripts are significantly different ($P \leq 0.05$).

Unlike the case with hypotaurine, there was a poor association between CDO protein and taurine levels in this study. In the liver, taurine levels fell significantly in LP rats. This was expected as CDO levels were depressed by the LP diet. What was not anticipated, however, was a gradual but significant decline in taurine from the livers of HP control rats by 10 h. This decline occurred in spite of high levels of hepatic CDO. While significant decreases in taurine were occurring in the liver, no significant changes were noted in the kidney for either the LP or HP groups. LP+CYS rats, which maintained high levels of hepatic CDO and elevated levels of kidney CDO, showed no change in either liver or plasma taurine but did exhibit a trend towards increasing taurine levels in the kidney. Taurine levels were significantly reduced in the LP+PII animals relative to both the LP and HP groups at all time points, despite having CDO levels comparable to the HP rats. The largest drop in taurine within this group (63%) occurred by 6 h, with no further decrease by 10 h. This decrease was accompanied by a significant increase in plasma and kidney taurine levels at 6 h that was eventually stabilized by 10 h.

Table 2. Effects of experimental diets and proteasome inhibition on taurine levels in liver, kidney, and plasma

	Time (h)	0 *[Fasting]*	6 *[Fed]*			
	Diet	*HP*	*LP*	*LP+CYS*	*LP+PII*	*HP*
Liver		13±0.9[a]	8±0.1[bd]	11±0.4[af]	4.5±1.7[cd]	11±0.4[af]
Kidney		8.4±0.4[a]	7.5±0.4[a]	8.7±0.5[a]	12±0.7[b]	8.5±0.2[a]
Plasma		66±4[a]	38±6[a]	62±7[a]	147±13[b]	45±10[a]
	Time *(h)*		10 [Fed]			
	Diet		*LP*	*LP+CYS*	*LP+PII*	*HP*
Liver			6.5±1[d]	10±0.3[af]	4.2±1[d]	9.7±1[f]
Kidney			8.4±0.6[a]	10.3±0.6[c]	8.7±0.2[a]	7.4±0[a]
Plasma			40±4[a]	55±12[a]	51±17[a]	38±9[a]

The unit for liver and kidney values is nmol/mg protein. The unit for plasma data is μmol/dl. The unit for plasma data is μmol/dl. Within a given tissue, values with different superscripts are significantly different ($P \leq 0.05$).

4. DISCUSSION

Although the ubiquitin-26S proteasome pathway is thought to be responsible for the half life of most if not all intracellular mammalian proteins, direct evidence for targeted ubiquitination and degradation has been gathered for only a handful of proteins - the majority being short-lived transcription factors and signal transduction molecules (Ciechanover, 2005; Ciechanover and Ben-Saadon, 2004). Studies involving these proteins have relied heavily upon evidence derived from *in vitro* cell-free or intact cell culture models, with no demonstration of physiological regulation of the queried protein by the ubiquitin-26S proteasome system in a living mammal. In this paper, however, we have shown that CDO - a protein involved in intermediary amino acid metabolism - is robustly regulated by the ubiquitin-26S proteasome system *in vivo* and that this regulation has a significant impact on flux through the cysteine sulfinate pathway.

Rats on the LP diet rapidly down-regulated CDO protein levels in response to low cysteine availability. This down-regulation was prevented by pharmacological inhibition of the proteasome. These results are consistent with what we have previously seen in cultured primary hepatocytes and transfected HepG2/C3A cells, which rapidly ubiquitinate and degrade CDO under conditions of low cysteine availability (Stipanuk *et al.,* 2004a). Ubiquitinated species of CDO were elevated in the livers of rats on the LP diet as well as rats on a LP diet that received a specific proteasome inhibitor, though the degree of ubiquitinated CDO accumulation was greater in rats receiving PII. The degradation of liver CDO was also prevented by increasing the dietary availability of cysteine. Again, this parallels nicely with the results of our previous cell culture studies, wherein hepatocyte CDO degradation was markedly attenuated upon incubation in a high cysteine medium. In the cell culture studies, ubiquitination of CDO was down-regulated in response to cysteine. We found a similar phenomenon occurring *in vivo*. The fraction of hepatic CDO as ubiquitinated species was significantly less in rats on the LP +CYS or HP diets compared to their LP or LP+PII cohorts for a given time point. We are currently researching how cysteine signals a change in the ubiquitination status of CDO. Additional work remains in identifying the ubiquitin ligase that docks with CDO and covalently modifies it with ubiquitin.

Prior to this study, efforts to understand the regulation of CDO have focused almost exclusively upon the liver. There are two principal reasons for this investigational bias: the liver is the first organ to be directly exposed to intestinally absorbed cysteine and the liver expresses CDO at concentrations that are orders of magnitude higher than other organs. An earlier study from our lab that evaluated the effects of a long-term (2 week) manipulation of sulfur amino acid intake in rats indicated no long-term changes in CDO protein within the kidney or other nonhepatic tissues (Stipanuk *et al.,* 2002). Our current results, however, suggest that CDO levels in the kidney can be acutely upregulated in response to ingestion of high cysteine (<12 h). The increase in CDO above what was observed in the HP groups may be attributable to the fact that the major sulfur amino acid in casein is methionine, supplementation of which will not increase intracellular cysteine levels as much as directly feeding cysteine to the animal. The modest increase in CDO levels following proteasome inhibition also suggests that renal CDO is regulated via its turnover by the ubiquitin-proteasome system. Because of the low levels of native CDO in the kidney, however, we were unable to directly confirm this hypothesis by detecting the less abundant ubiquitinated species of CDO for any of the treatment groups.

Catalyzing the first step in the cysteine→taurine pathway, CDO is strategically positioned for regulating the synthesis of hypotaurine and taurine. We predicted that an increase in CDO protein, either through the administration of cysteine or PII, would increase flux through the cysteinesulfinate pathway and result in the accumulation of these two amino acids. We did not look for the accumulation of cysteinesulfinate due to the fact that endogenous levels are kept low *in vivo* by the combined actions of cysteinesulfinate/aspartate aminotransferase and cysteinesulfinate decarboxylase (unpublished results). Hypotaurine levels responded in accordance with our prediction, increasing in liver and kidney. Intracellular taurine levels, on the other hand, were not always directly associated with tissue CDO levels. Most notably, taurine levels were actually decreased by proteasome inhibition. The marked increase in taurine plasma levels associated with PII suggests that it may have activated taurine efflux pathways. Variability in taurine levels within the HP groups, however, coupled with the competing use of taurine for osmotic balance and bile acid synthesis (Huxtable, 1992) suggests that using steady-state levels of taurine to estimate cysteine sulfoxidation flux over the time-scale of this study may not be appropriate. Steady-state levels of hypotaurine, which is found at concentrations almost 100-fold lower than taurine and is not involved in any known physiological process apart from taurine synthesis, appear to be a more accurate reflection of flux. Indeed, the levels of this cysteine metabolite have been previously shown to be closely tied to sulfoxidation flux (Kasai *et al.,* 1992).

In summary, our results show that CDO levels within the liver and kidney are actively regulated by the 26S proteasome system. Altering the level of CDO by manipulation of the ubiquitin-proteasome system dramatically affected steady-state levels of hypotaurine, demonstrating that *in vivo*, CDO is an important regulator of cysteine sulfoxidation flux.

5. ACKNOWLEDGMENTS

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OSMOSENSITIVE GENE EXPRESSION OF TAURINE TRANSPORTER AND CYCLIN C IN EMBRYONIC FIBROBLAST CELLS

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1. ABSTRACT

Hypertonic conditions induce osmoregulatory activity. Molecular mechanism, however, remains to be further elucidated concerning the osmosensitive balancing activity. Using a differential display protocol, the genes of taurine transporter (TauT) and cyclin C were identified as candidate responding to the hypertonicity. When a quantitative PCR analysis was performed on the total RNA from KBEF cell treated under hypertonic conditions, the gene expressions of TauT and cyclin C were greatly increased. In terms of protein, the level of TauT expression increased up to 3.2-fold in response to the hypertonic treatment. Similarly to TauT, cyclin C protein also increased 2.4-fold compared to the control treatments. Under taurine-rich extracellular conditions, however, the level of TauT expression increased as little as 1.8-fold by hypertonicity treatment. Cyclin C expression was also lowered compared with low-taurine hypertonic treatment. Cysteine dioxygenase was shown to be highly responsive to the hypertonic treatment. Taken together, these results strongly indicate that the gene expressions of TauT and cyclin C are cooperatively regulated under hypotonic conditions.

Key words: hypertonicity, taurine, cyclin, cysteine dioxygenase

2. INTRODUCTION

Hypertonicity exerts a great challenge to cellular homeostasis. To cope with an increase in extracellular osmolality, living organisms as diverse as bacteria and mammals

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utilize intracellular non-perturbing osmolytes. In mammalian cells, the extracellular hypertonicity is balanced by retaining a high intracellular content of organic osmolytes like betaine, glycerophosphorylcholine, *myo*-inositol, sorbitol, and taurine (Jones *et al.*, 1995; Berg *et al.*, 1997; Kang *et al.*, 2002). Among these osmolytes, taurine serves as a prime role in balancing osmolarity to maintain the homeostatic system. Hypertonicity induced the activity of these osmolyte transporters (Satsu *et al.*, 2003, 2004), this osmo-sensitive response being used to maintain the cell volume against exposure to the hypertonic environment. Indeed, hypertonicity has been known to up-regulate membrane transporters' activity for these osmolytes (Takashi *et al.*, 2004). Other than increasing membrane transporter's function, hypertonicity also induces expression of various genes when countered with a significant change in extracellular osmolality to inhibit cell division.

The osmoregulation of the osmolyte transporters has been studied in several lines of tissues focusing upon osmotic regulation of osmolyte memebrane transporters (Lima *et al.*, 1998; Bitoun *et al.*, 2001). Among the cell lines used, kidney cells were extensively investigated since the kidney medulla is a typical tissue that normally becomes hypertonic via the urinary concentrating mechanism. In this study, the gene expression is studied as an osmotic response in the embryonic fibroblasts that have not previously been investigated. Among various types of epithelial cells, intestinal epithelial cells are routinely exposed to hypertonic conditions when the digested food substances come into the luminal tract of the small intestine. In fact, it has been reported that the volume of intact crypts isolated from the guinea-pig small intestine was changed by exposure to hypertonicity. However, it is not known whether or not some of the organic osmolytes work to maintain the volume of intestinal epithelial cells.

Numerous genes are modulated in their expression under hypertonic conditions. Only a minority of these genes has been identified as cell cycle genes to date (Uchida *et al.*, 1992; Itoh *et al.*, 2004; Tappaz, 2004). Only a few cell cycle-regulated genes and the cyclin-dependent kinase are considered as candidates. Cell cycle progression from G1 to S phase is regulated by phosphorylation of proteins by cyclin/CDK complexes. CDKs are serine-threonine kinases that drive the cell cycle through phosphorylation of a number of key substrates (McManus and Strange, 1993). In the present study, the hypertonicity-induced change in the gene expression of taurine transporter and cell cycle genes was studied using the fibroblast embryonic cell line. Following treatment with hypertonicity, differential gene expression was analyzed by the differential display polymerase reaction (DD-PCR) procedures.

This study was designed to identify genes that are modulated under hypertonicity. When cells were exposed to hypertonicity, the differential display procedure revealed that the taurine transporter (TauT) and cyclin C were shown to be upregulated among the genes responding to the hypertonic treatment. Analysis of mRNA and protein expression confirmed that they serve as primary genes responding to the hypertonicity. These gene products may serve as prime functional factors to cope with hypertonicity.

3. MATERIALS AND METHODS

3.1 Compounds and Hypertonic Cell Culture Conditions

Taurine ($NH_2CH_2CH_2SO_3H$, MW 125.1) was purchased from Sigma Chemicals (St.

Louis, USA). Korean bovine embryonic fibroblast (KBEF) cells were maintained routinely at 36.5°C in DMEM supplemented with 2 mM L-glutamine, 100 U/ml penicillin-streptomycin and 5% fetal calf serum (FBS). The hyperosmotic medium (600 mOsmol/kg) was prepared by adding NaCl and urea into the isotonic medium (Kasano *et al.*, 2005). Urea is a permeable solute and does not create an osmotic gradient. Supplementation of urea in the media is based on good viability and stability of cultured cells under long-term exposure to hypertonic conditions. Hypertonicity was achieved by adding NaCl and urea as 10:1 ratio per weight basis. The hypertonic medium was prepared up to 600 mOsmol/kg.

3.2. Differential Display Polymerase Chain Reaction

Total RNAs were isolated by the RNAzol^{TM} (TEL-TEST, Inc.) procedure and DNA contaminants were eliminated by RNase-free DNase I (Promega, Madison, USA). M-MLV reverse transcriptase (Promega) was used to synthesize first-strand cDNAs in the presence of 10 μg of RNA, 0.25 μg of oligo(dT_{11}) primer ($AAGCT_{11}G$), 0.5 mM each of dNTP, and first strand buffer 25 mM Tris-HCl (pH 8.3), 37.5 mM KCl, 1.5 mM $MgCl_2$, 5 mM DTT, and 200 U of the reverse transcriptase as in a final volume of 20 μl for 60 min at 37°C. Following ethanol precipitation, the cDNA was subjected into a PCR reaction mixture including 2 μM downstream primer, 1 μM upstream primer (ttgcaagattgcg), 2 mM dNTPs, 1.8 mM $MgCl_2$, and 1 U Taq polymerse in a total volume of 30 μl. Following initial denaturation for 5 min (94°C), the PCR cycle included the thermal cycle of 30 s (94°C)->1 min (42°C)->1 min (72°C) for 50 cycles. The PCR products were electrophoresed on a 6% non-denaturing polyacrylamide gel in a TBE buffer. The gel was then subjected to silver staining. The bands showing different expression patterns were excised and eluted in H_2O at 90°C for 5 min. Candidate DNA was re-amplified in the presence of 2 μM upstream primer, 5 mM dNTPs, 1.5 mM $MgCl_2$, and 1 U Tag polymerase in a total volume of 50 μl with the same PCR profile as above. The amplified cDNA was subcloned into pGEM-T and sequenced.

3.3. Quantitative RT-PCR

In order to maintain the PCR reaction within its linear range, a limiting cycle amplification protocol was utilized. First-strand cDNA synthesis was performed on total RNA (2 μg) using M-MLV reverse transcriptase. PCR amplification was performed using this cDNA as a template in a PCR reaction with thermal cycle profile of 1 min (95°C), 2 min (55°C), and 30 s (73°C) for 4 cycles and then 10 s (95°C)->1 min (55°C)->30 s (73°C) for 25 (cyclin C), 30 (TauT), or 20 (β-actin) cycles, respectively. The following specific primer pairs were used: TauT, 5'-atttccttcttttggtttttcagcc-3' and 5'-agatgagccaaca-cagccagtaa-3'; cyclin C, 5'-cagcaatgcatcctgcacc-3', and 5'tggactgtggtcatgagccc-3'; actin 5'-tgaaaagatgagtatgcctg-3' and 5'-ccaacctgctcagatacatc-3'. Cysteine dioxygenase (CDO) expression was quantitated according to Qusti *et al.* (2000). Amplified PCR products were subsequently 5'-end-labeled using SyberGreen. Using SyberGreen chemistry, the amplified product was detected by its interaction with the SyberGreen fluorescent dye according to Martinez *et al.* (2002). The ratio of PCR products and unincorporated primers was normalized to the respective actin PCR product/primer ratio, thus providing a value for relative mRNA expression of TauT or cyclin C.

3.4. Western Blotting Analysis

Cells were harvested and sonicated in a cold lysis buffer (50 mM Tris-HCl, pH 7.5, 0.1% SDS, 0.1 mM leupeptin, 0.5 mM PMSF). Fifty microgram of protein was subjected to SDS/PAGE and transferred to Protran membrane (Schleicher & Schuell, Keene, USA) according to the semi-dry method at 15V for 30 min at room temperature in transfer buffer (25 mM Tris-HCl, pH 8.5, 0.2 M glycine, 20% methanol). Membranes were blocked in 10% milk-PBST buffer (1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4, 150 mM NaCl, 2.7 mM KCl, 0.05% Tween-20) for 1 h at 25°C. TauT was detected with rabbit anti-human antibody (1:1,000) (Santa Cruz Biotechnology, Santa Cruz, USA) while cyclin C was probed with rabbit anti-human polyclonal antibody (1:5,000) (Santa Cruz Biotechnology). Membranes were incubated overnight at room temperature with mild shaking in PBST containing 10% low-fat milk. Following sufficient washing with PBST, the immunoblots were further incubated for 1 h at room temperature with either a secondary horseradish peroxidase conjugated anti-rabbit IgG antibody in PBS-T containing 10% low fat milk, or a secondary anti-goat IgG. Subsequent ECL detection was performed according to the manufacturer's recommendations (Amersham, Piscataway, USA). The levels of protein expression were quantified by the use of a Packard Cyclone reader. Equivalent loading of protein samples was ensured by immunodetection with an anti-actin polyclonal antibody and additional Coomassie blue staining of the respective blots.

4. RESULTS

4.1. Isolation of Primary Hypertonicity Responding Genes

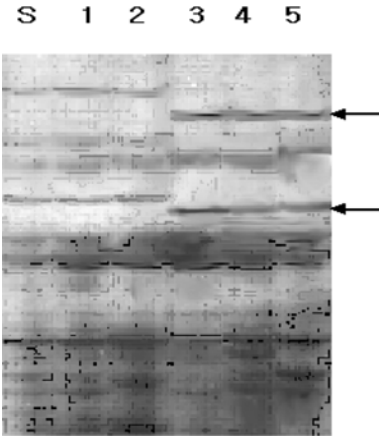

Figure 1. DD PCR on total RNA from KBEF. Hypertonicity induced genes are detected at low RNA levels from KBEF treated with osmorality ranging 50-700 mOsmol/kg for 2 hours. Total RNA was used to prepare cDNA and DD patterns were obtained. The arrows indicate cDNAs up-regulated under hypertonic conditions. The upper arrow refers to cyclin C and lower, to TauT. Lanes 1-5 refer to 50, 100, 450, 600, 700 mOsmol/kg in the media, repectively, while lane S represents the incubation using 0.7 % NaCl in the media.

The gene expression pattern was analyzed according to the differential display method on the total RNA isolates from KBEF cells treated with osmolality up to 600 mOsmol/kg for 2 h. The differential display analysis revealed that many genes varied their expression under the hypertonicity treatment. Several of the cDNA bands representing gene transcripts disappeared or decreased in KBEF, whereas others increased or were exclusive to the latter. Fig. 1 shows such bands with varied intensity patterns compared to the control lanes. Fifteen bands were recognized using 9 different combinations of upstream and downstream primers. Six promising bands were selected based on the band intensity. Each DNA was isolated, subcloned and sequenced. Sequence data indicated taurine transporter and cyclin C as genes responding to components in hypertonicity. Other candidate genes are as yet unidentified or insignificant and omitted from further study.

4.2. Quantitative Assessment of mRNA Expression by RT-PCR

Quantitative PCR analysis of TauT showed that TauT transcription was significantly up-regulated by hypertonicity in KBEF cells. KBEF cells were treated under the hypertonic conditions and total RNA was isolated following various treatment periods. The total RNA extracts was reverse-transcribed into cDNA and subjected to the quantitative PCR. The relative mRNA expression is calculated as the ratio of each expression against control and normalized for actin mRNA expression. Fig. 2 shows the time course of cyclin C and TauT mRNA expression in response to the hypertonicity treatment. KBEF cells were treated for 1, 2, 4, and 24 h with hypertonicity and were compared with control cells which were incubated in PBS for 2 h. TauT mRNA expression appeared to be upregulated by hypertonicity (Fig. 2A). Cyclin C mRNA was also up-regulated with hypertonicity (Fig. 2B). This consistent expression pattern in the two osmosensitive genes strongly imply that taurine may be the major functional osmolyte and expression of TauT and cyclin C are cooperatively regulated responding to the hypertonicity.

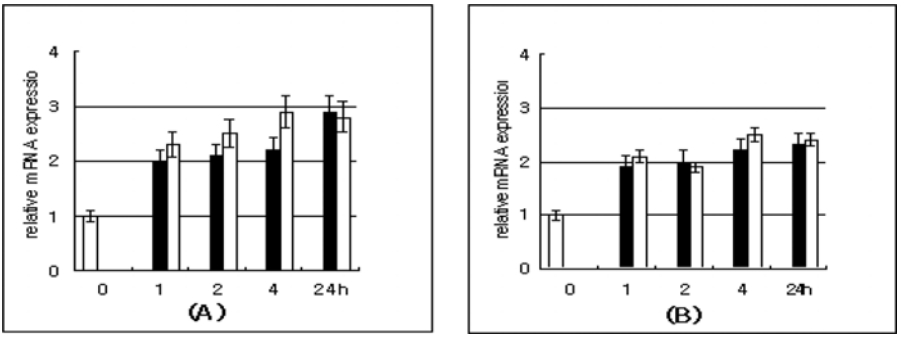

Figure 2. Time course of TauT and cyclin C mRNA expression in response to hypertonicity. KBEF cells were treated for 1, 2, 4, and 24 h under hypertonic conditions. Relative gene expression was compared with non-treated cell (0 h). Hypertonicity was generated by adding NaCl at two concentrations: 450 mOsmol/kg (*dark bars*) and 600 mOsmol/kg (*light bars*). RT-PCR was performed with primer pairs that were specific for the taurine transporter (A) and cyclin C (B) genes using total RNA. The relative mRNA expression is calculated as the ratio of each expression.

4.3. Immunoblotting

The altered gene expression was analyzed at the protein level by immunoblotting according to the time (Fig. 3). As the control for loading, actin was used as the quantity marker. The relative protein expression was calculated as the ratio of protein expression for the non-treated and the actin signal. The results showed that cyclin C expression was greatly increased during hypertonic treatment. This increase also coincides in TauT and cyclin C. These results indicate that components in hypertonicity not only increased the mRNA expression but these genes lead to the increased expression of the protein. Most likely taurine is the major functional component in response to the osmotic change.

Relative protein expression was measured for TauT, cyclin C, and cysteine dioxygenease (CDO) following the hypertonicity or taurine treatment. In many studies, CDO has been well-characterized as its expression is highly correlated with TauT under hypertonic conditions (Qusti *et al.,* 2000; Satsu *et al.,* 2003). As expected, the protein expression pattern was consistent for TauT and CDO. For the proteins tested in this study, cyclin C expression increased 2-4 fold when compared to the control experiment. The expression level of the three proteins decreased when the extracellular concentration of taurine increased under the hypertonic treatment. These findings indicate that hypertonicity modulates cell cycle-regulating genes. When countered with the hypertonic conditions, taurine plays an important role in modulating the intracellular osmolarity.

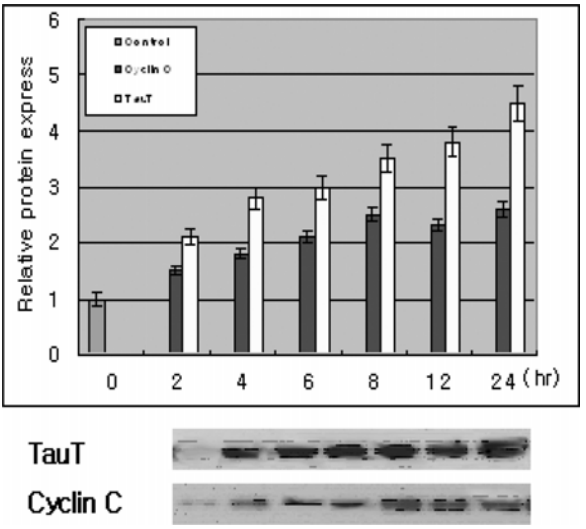

Figure 3. TauT and cyclin C expression in response to hypertonicity. KBEF cells were treated for 4, 6, 8, 12, and 24 h under osmolarity of 600 mOsmol/kg. Equal amount of total cellular extract were separated through a 10% SDS-PAGE and electroblotted to perform immunoblotting. TauT (light bars) and cyclin C (dark bars) protein expression was assessed with a primary antibody and ECL detection. Control (gray at time 0) refers to a 2-hour incubation in the 0.7% saline. Values obtained for the treatments are expressed as fold induction of control treatments and corrected according to the actin expression level.

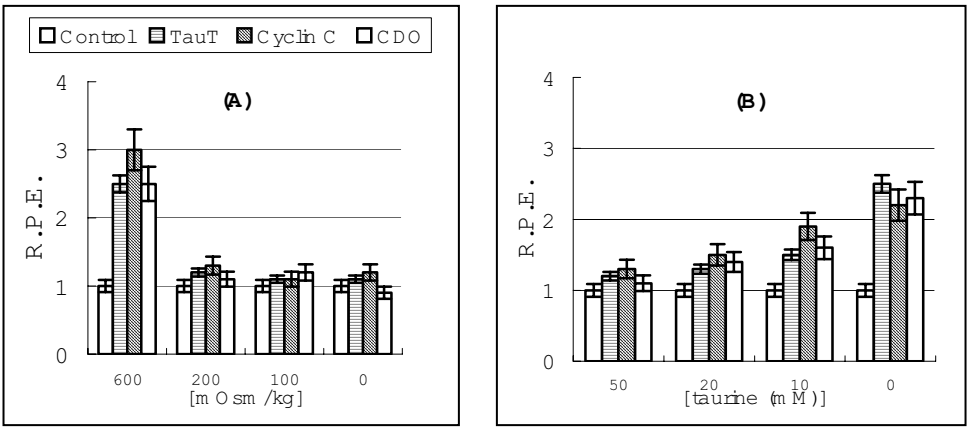

Figure 4. Osmosensitive protein expression under hypertonic conditions. (A) After cells were treated for 24 h under various osmotic conditions, the protein expression was compared among the four conditions. R.P.E. (relative protein expression) refers to standardized values considering actin expression level as 1. Equal amounts of total cellular extracts were electrophoresed on a 10% SDS PAGE. Control and CDO refer to actin and cysteine dioxygenase, respectively. (B) Cells were incubated with the extracellular taurine concentrations of 0-50 mM. Using same symbols as in (A), THE protein expression was quantified by fluoroimaging analysis and normalized to actin (control) protein expression. Values of relative expression were obtained as fold induction against control treatments. Columns refer to mean values of Western blots (n=10); bars to standard deviations (SD).

5. DISCUSSION

This study was aimed to analyze gene expression in response to hypertonicity or taurine in the cells of KBEF. The genes of TauT and cyclin C were initially identified as candidates using a differential display approach. The analysis of mRNA and protein expression confirmed that TauT and cyclin C act as primary osmosensitive genes responding to the hypertonicity in KBEF cells. The gene expression for TauT and cyclin C was greatly augmented in the hypertonicity-treated KBEF cell.

It may be a challenge to understand the simultaneous up-regulation of cyclin C and TauT under hypertonicity or taurine treatment. To date, no report is available that cyclin C expression affects TauT expression or *vice versa*. The two genes might have a common upstream responsive element responding to a change in the extracellular osmolality. Further study is necessary to identify the upstream regions or to locate transacting elements whose expression is modulated by hypertonicity. Cyclin C belongs to the cyclin family of proteins that control cell cycle transitions through activation of specific catalytic subunits, the cyclin-dependent kinase 8 (cdk8). The periodic degradation of typical cyclins is crucial for cell-cycle progression and depends on the catalytic activity of the associated CDK. The up-regulation of cyclin C upon hypertonicity treatment may induce expression of genes for cell growth arrest, differentiation and even the programmed cell death. This may provide a new insight into the mechanisms of cell cycle gene products leading to general transcriptional regulation affected by hypertonicity and/or taurine. This study shows that hypertonicity and taurine are effective enough in KBEF cell lines to induce differential gene expression. Both factors are able to impede the progression of cell cycle.

Cells retain an orderly sequence of events in which they maintain their contents and then divide in two during the cell cycle. In most cells, additional steps of regulation enhance the fidelity of cell division and allow the control system to respond to various extracellular signals. The essential features of this system consist of an ordered succession of biochemical events that undergo the cell cycle sequence including DNA replication and the segregation of the replicated chromosomes. Under hypertonic conditions, cells are required to keep the cellular integrity before long and complex sequences of cell divisions occur. When countered with hypertonocity, cell division should be blocked and eukaryotic cells have evolved a complex network of regulatory proteins, known as the cell cycle control system. Cyclin C may serve as a switch on the cell cycle control system to modulate cellular activity in the face of hypertonicity.

Human cells are highly responsive to signals from outside concerning cell division progression. When countered with hypertonicity, the control system delays progression of the cell cycle until the unfavorable extracellular conditions have been removed. Hypertonicity-induced up-regulation of cyclin C may inhibit cell cycle progression; thus, it has a central role in regulating cell size and numbers. Delayed onset of cell cycle may be achieved via cyclin C up-regulation. Under circumstances, the segregation of replicated chromosomes is not permitted until the hypertonicity is cleared. This observation may add cyclin C to the list of genes responding to hypertonicity. These results also suggest that osmotic regulation may be achieved through the concerted action of TauT and cell cycle-related genes.

Taken together, the modulation of cell-cycle genes seemed to be a major mechanism responsible for the growth-inhibitory effect under hypertonic conditions. Along with the up-regulation of TauT, this study provides fresh insight into the mechanisms of cell cycle and general transcriptional regulation by components in hypertonicity or taurine. Based on the enhanced expression corresponding between taurine and hypertonicity treatments, we suggest that taurine is the prime functional osmoregulator to cope with hypertonicity. In addition, a direct comparison may not be feasible in gene induction potential between taurine and cyclic C. The coinciding alterations in the level of cyclin C and TauT expression seems implies that their gene expression might be coordinated by a common transcriptional factor.

6. ACKNOWLEDGMENTS

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IS *TauT* AN ANTI-APOPTOTIC GENE?

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1. INTRODUCTION

Recent studies have demonstrated that taurine and the taurine transporter play an important role in kidney development (Heller-Stibl *et al.*, 2002; Han *et al.*, 2000). In the offspring of inbred taurine-deficient cats, taurine deficiency results in renal malformation with significantly diminished renal size and progressive kidney damage. Histological examination of these kidneys showed ureteral dilatation, enlarged glomeruli, proximal tubular flattening and abnormal differentiation of renal tubular epithelium, especially in the distal tubule, where taurine has been found to be in highest concentration in immunohistochemical studies. Consistent with these findings, Heller-Stilb *et al.* (2002) demonstrated that knockout of *TauT* resulted in severe and progressive retinal degeneration, a small brain, and shrunken kidneys in a *TauT -/-* mouse model. These findings confirm that *TauT* is required for retinal, brain, and kidney development.

2. MATERIALS AND METHODS

2.1. Construction of the Reporter Gene

In this study, we generated a pGL963-reporter gene construct by using a 1.1 kb fragment of the *TauT* promoter region DNA as the template for PCR (GenBank accession number AR151716) and the PCR fragment was cloned into the promoterless luciferase vector pGL3-Basic or SV40/pGL-3 control vector (Promega, Madison, WI). The conditions used were 30 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 58°C, and 1 min of elongation at 72°C. The sense primer (5'-GGGGTACCTTAC-TGAAGGTC-ACACAGC-3') designed for PCR contained a unique site for *KpnI*, and the antisense primer (5'-AAGATCTTGGCACGGGAG-TTCA-3') contained a unique site for *BgI II*. PCR products were digested with *KpnI* and *BgI II* and re-ligated into *KpnI* and *BgI II* sites of pGL3-Basic or pGL3-control vectors.

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2.2. Cell Culture

Wild-type and *TauT*–overexpressing LLC-PK1 cells were cultured according to ATCC (American Type Culture Collection) guidelines. Briefly, cells are grown as confluent monolayers in 10 cm diameter tissue culture plates in media specific for each cell line with 10% fetal calf serum at 37ºC in the presence of 5% CO_2 in a humidified incubator. For experiments, 1×10^6 cells were seeded and cultured overnight, and 80% confluent cells were used.

2.3. Transient Transfection

Plasmid DNA was introduced into cultured mammalian cells using SuperFect Transfection Reagent (Qiagen). Transfection was carried out for 16-18 h, and then cells were washed twice with phosphate-buffered saline and incubated in fresh medium for 24-48 h before harvesting. pGL-control, which contains a luciferase gene driven by the SV40 early region promoter/enhancer, and empty pGL-Basic vectors were used as positive and negative controls, respectively. To standardize the transfection efficiency, 0.1 μg of pRL-CMV vector (pRL Renilla Luciferase control reporter vector, Promega) was cotransfected in all experiments. Cells were harvested 48 h after transfection and lysed in 200 μl of reporter lysis buffer (Promega). A luciferase assay was performed using a dual-luciferase assay kit (Promega), and activity was measured with an Optocomp 1 luminometer (MGM Instruments, Inc., Hamden, CT). Promoter activity (mean ± SD of four samples in relative light units) of each construct is represented by relative light output normalized to pRL-CMV control. Graphs represent typical results of four separate experiments. The concentration of protein in the cell extracts was determined using the Bradford method (Bio-Rad, Hercules, CA).

2.4. Measurement of Taurine Transport

Taurine transport studies were performed on confluent monolayers 3 days after seeding cells. Briefly, cells were washed with Earle's Balanced Salt Solution (EBSS) at 37°C. Uptake was initiated by the addition of uptake buffer (2 mM KCl, 1 mM $MgCl_2$, 96 mM NaCl, 1.8 mM $CaCl_2$, 5 mM Hepes, pH 7.6) to which 50 μM unlabelled taurine and 0.5 μCi/ml [^{14}C]taurine (Perkin Elmer, Boston, MA) were added. After incubation for 30 min at room temperature, uptake was terminated by the removal of uptake buffer followed by three rapid washes with cold EBSS. Cells were solubilized in 1% SDS in 0.2 N NaOH and radioactivity counted in a Packard 2000-CA Liquid Scintillation Analyzer.

2.5 Western Blot Analysis

Cells were lysed in 50 μl M-PER mammalian protein extraction reagent (Pierce, Inc., Rockford, IL) supplemented with a protease inhibitors cocktail for use with mammalian cell and tissues extracts (Sigma, St. Louis, MO). The lysates were cleared by centrifugation at 14,000 × *g* for 2 min, and the supernatants transferred to clean tubes. Equal amounts of protein (50 μg) were separated by electrophoresis on a 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Millipore, Bedford, MA) using a semi-dry electrophoretic transfer system (Bio-Rad, Hercules, CA). Membranes were incubated in 5% nonfat dry milk in Tris base/sodium chloride (TBS)

buffer with 0.2% Tween 20 (TBST) at 4°C overnight. The membranes were incubated with primary antibody for 1 h at room temperature, washed with TBST and incubated with horseradish peroxidase-linked secondary antibody (Sigma) for 1 h, and then the proteins of intereste were detected using a chemiluminescent detection kit (Pierce, Inc.).

2.6. Apoptosis Assays

Internucleosomal DNA fragmentation was detected primarily by DNA laddering assay following the manufacturers' instructions. Briefly, equal numbers of cells were resuspended in 500 µl of lysis buffer (1% sodium dodecyl sulfate, 25 mM ethylenediaminetetra acetic acid, and 1mg/ml proteinase K) and incubated overnight at 50ºC. Ribonuclease A (10 mg/ml) was then added for an additional 2 h incubation at 37ºC. The chromosomal DNA was extracted with phenol/chloroform, precipitated with ethanol, and analyzed by 1% agarose gel electrophoresis followed by staining with ethidium bromide to reveal the fragmentation pattern.

2.7. Materials

[^{14}C]Taurine (92.1 Ci/mol) was purchased from Du Pont, Perkin-Elmer. LLC-PK1 cells were purchased from ATCC and cisplatin was purchased from Sigma.

2.8. Statistics

All experiments using tissue cultures were performed in triplicate. Luciferase assay results were expressed in units of relative light output. Statistical comparisons were made using one-way ANOVA and Student's *t* test to determine significant differences in the means between experimental groups.

3. RESULTS

3.1. Down-Regulation of Taurine Transport by Cisplatin in LLC-PK1 Cells

To determine if cisplatin affects the function of the taurine transporter, LLC-PK1 cells were treated with or without cisplatin (0-100 µM) for 24 hours and taurine transport activity by the cells was measured as described in the Methods. As shown in Fig. 1, cisplatin significantly decreased the taurine transport activity of LLC-PK1 cells in a dose-dependent manner. The amount of taurine uptake by control cells (untreated) was 1.56 ± 0.12 nmol/mg protein, and 0.97 ± 0.05 nM/mg protein by cisplatin (100 µM)-treated cells.

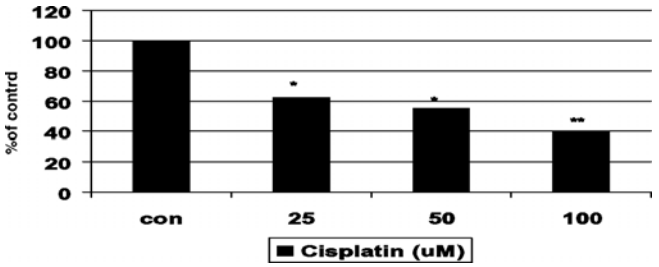

Figure 1. Effect of cisplatin on taurine uptake. LLC-PK1 cells were cultured in DMEM/F12 medium containing cisplatin as indicated for 24 h, and then taurine uptake was measured. * $p<0.05$ *vs*. control, ** $p<0.01$ *vs*. control.

3.2. Down-Regulation of *TauT* Expression by Cisplatin

To determine whether cisplatin represses expression of the *TauT* gene, Western blot analysis was performed in cells treated with cisplatin (25-100 μM) for 24 hours using a specific *TauT* protein antibody produced by our laboratory. As shown in Fig. 2, expression of *TauT* was down-regulated by cisplatin in a dose-dependent manner.

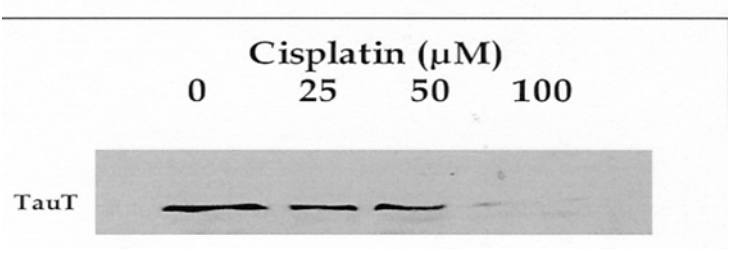

Figure 2. Dose-dependent regulation of *TauT* by cisplatin. LLC-PK1 cells were cultured in medium containing cisplatin for 24 h, and then Western blot analysis of *TauT* was carried out.

3.3. Transcriptional Repression of *TauT* by Cisplatin

To determine if down-regulation of the *TauT* gene by cisplatin occurs at the transcriptional level, the reporter construct pGL-963 was transiently transfected into LLC-PK1 cells, and regulation of *TauT* promoter function by cisplatin was examined. As shown in Fig. 3, cisplatin decreased *TauT* promoter activity in LLC-PK1 cells in a manner similar to that observed in Figs. 1 & 2, suggesting that cisplatin represses *TauT* expression at the transcriptional level.

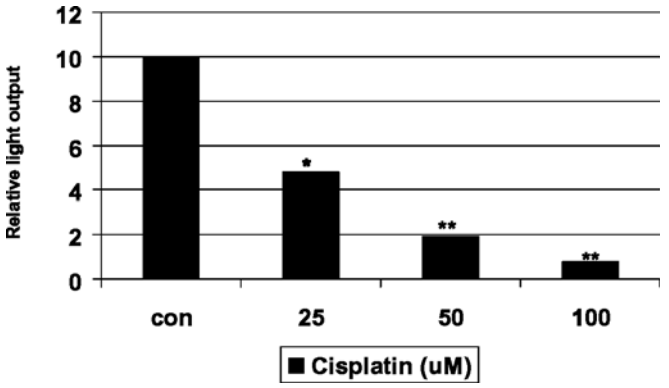

Figure 3. Down-regulation of *TauT* promoter activity by cisplatin in renal cells. pGL-963 was transiently transfected into LLC-PK1 cells treated with cisplatin for 24 h . The luciferase assay was performed using cell lysates. * $p<0.05$ *vs*. control, ** $p<0.01$ *vs*. control.

3.4. Up-Regulation of p53 by Cisplatin in LLC-PK1 Cells

To examine whether cisplatin would induce expression of endogenous p53, Western blot analysis was carried out in cells treated with cisplatin for 24 hours. We found that expression of p53 was induced by cisplatin in LLC-PK1 cells in a dose-dependent manner (Fig. 4). This result suggests that cisplatin down-regulates *TauT* expression, at least in part, through a p53-dependent pathway.

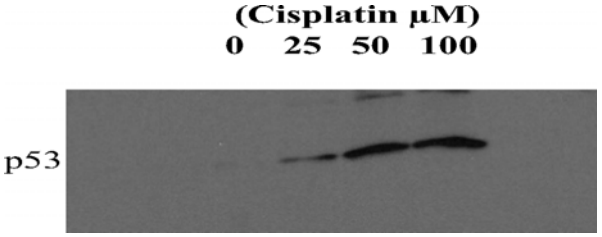

Figure 4. Cisplatin-induced p53 expression in LLC-PK1 cells. Cells were cultured in medium containing cisplatin for 24 h, and then Western blot analysis of p53 expression was carried out.

3.5. Effect of Forced Overexpression of *TauT* on Taurine Uptake

Studies have shown that cisplatin induces dose- and duration-dependent apoptosis in LLC-PK1 cells (Park *et al.*, 2002). To test if *TauT* functions as an anti-apoptotic gene in

LLC-PK1 cells, *TauT* cDNA was stably transfected into the LLC-PK1 cells selected by G418. To test whether forced overexpression of *TauT* blocks the effect of cisplatin on taurine uptake by LLC-PK1 cells, wild-type and *TauT*-overexpressing LLC-PK1 cells were treated with cisplatin (50 μM) for 24 hours, and taurine uptake was measured. The untreated cells and cells stably transfected with the vector alone were used as controls. As shown in Fig. 5, taurine uptake by wild-type LLC-PK1 cells and cells transfected with vector alone was decreased by more than 50% after treatment with cisplatin, as compared to the amount of taurine taken up by control cells. Overexpression of *TauT* doubled the taurine uptake and attenuated the effect of cisplatin on taurine uptake in *TauT*-overexpressing cells, as compared to control cells.

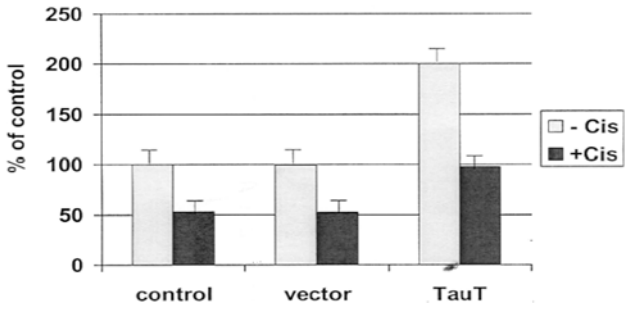

Figure 5. Effect of cisplatin on taurine uptake. *TauT* cDNA was stably transfected into LLC-PK1 cells. Cells transfected with vector alone were used as a negative control. Cells were cultured in medium containing cisplatin (50 μM) for 24 h, and then taurine uptake was measured.

3.6. Effect of Forced Overexpression of *TauT* on Cisplatin-Induced Apoptosis

To determine if forced overexpression of *TauT* attenuates cisplatin-induced apoptosis, wild-type and *TauT*-overexpressing LLC-PK1 cells were treated with cisplatin (0-100 μM) for 24 h, and DNA fragmentation was analyzed by DNA ladder following the manufacturer's instructions (R & D systems, Minneapolis MN). As shown in Fig. 6, the DNA laddering was detectable in cells treated with 50 μM cisplatin (lane 3) and was clearly evident in the 100 μM cisplatin-treated wild-type cells (lane 4).

However, DNA laddering was not observed after treatment with 50 μM cisplatin (lane 7) and was significantly reduced in the presence of 100 μM cisplatin in *TauT*-overexpressing LLC-PK1 cells (lane 8). These results indicate that the apoptotic effects of cisplatin are dose-dependent and can be attenuated by forced overexpression of *TauT*.

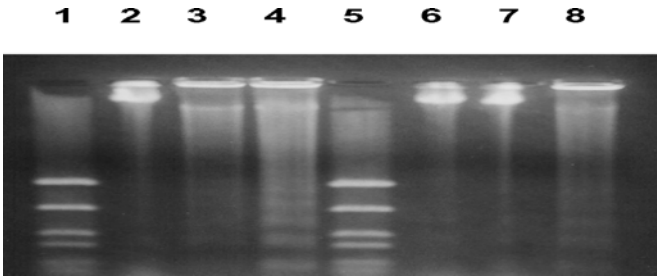

Figure 6. Effect of forced overexpression of *TauT* on cisplatin-induced LLC-PK1 cell apoptosis. Wild-type and *TauT*-overexpressing LLC-PK1 cells were cultured in medium containing cisplatin for 24 h, and then apoptotic DNA ladders were performed. Lane 1, DNA standard; Lane 2-4, wild-type LLC-PK1 cells with cisplatin 0, 50, and 100 μM; Lane 5, DNA standard; Lane 6-8 *TauT*-overexpressing LLC-PK1 cells with 0, 50, or 100 μM cisplatin.

4. DISCUSSION

Cisplatin is one of the most widely used chemotherapeutic agents for the treatment of several human malignancies. Nephrotoxicity after cisplatin treatment is common and may manifest after a single dose with acute renal failure or may present with a chronic syndrome of renal electrolyte wasting. The cytotoxic effects of cisplatin are found to occur via several mechanisms, including inhibition of protein synthesis, DNA damage and mitochondrial injury (Brady *et al.*, 1990; Huang *et al.*, 1995), which lead ultimately to activation of programmed cell death pathways in tumor cells and renal tubule cells (Megyesi *et al.*, 1998; Takeda *et al.*, 1998; Lau *et al.*, 1999; Okuda *et al.*, 2000; Ueda *et al.*, 2000).

Studies have shown that taurine can prevent cell apoptosis through several mechanisms, including inhibition of the generation of reactive oxygen species (ROS), nitric oxide (NO), tumor necrosis factor alpha (TNF-alpha), and regulation of intracellular calcium flux (Seabra *et al.*, 1998; Gurujeyalakshim *et al.*, 2000; Huang *et al.*, 2003). However, such protective actions of taurine require the normal function of the taurine transporter located on the cell membrane (Maar *et al.*, 1998). These findings led us to postulate that the normal function of the taurine transporter is impaired in cisplatin-induced renal injury, and cisplatin-induced nephrotoxicity may be prevented by increased expression of the *TauT* gene.

Elevated levels of p53 have been found in the kidneys of animal models of acute renal failure induced by cisplatin administration (Saad *et al.*, 2002). Negative regulation of *TauT* gene expression by p53 may play a role in the action of cytotoxic drugs, such as cisplatin-induced renal failure. Cisplatin accumulates in cells from all nephron segments but is preferentially taken up by the highly susceptible proximal tubule cells within the S3 segment (the site for renal adaptive regulation of *TauT*, which bear the brunt of the damage (Leibbrandt *et al.*, 1995; Matsell *et al.*, 2002). A recent study showed that taurine was able to attenuate cisplatin-induced nephrotoxicity and protect renal tubular cells from tubular atrophy and apoptosis (Smith *et al.*, 1992).

The promoter region of the taurine transporter gene contains a consensus binding site for the p53 tumor suppressor gene, which functions as a cell cycle checkpoint, blocking

cell division in the G1 phase to allow repair of damaged DNA or even triggering apoptosis in cells that have defective genomes (Hartwell *et al.*, 1994). Numerous stimuli trigger increases in the level of p53 expression, including DNA-damaging drugs, ionizing radiation, ultraviolet light, and hypoxia (Pei *et al.*, 1999; Ashcroft *et al.*, 2000; Hirao *et al.*, 2000; Nylander *et al.,* 2000). Varmus' group has found that transgenic mice over-expressing p53 undergo progressive renal failure through a novel mechanism by which p53 appears to alter cellular differentiation, rather than by growth arrest or the direct induction of apoptosis (Godley *et al.*, 1996). These findings suggest that altered expression of certain p53 target gene(s) involved in renal development may be responsible for p53-induced progressive renal failure in p53 transgenic mice.

In this study we demonstrated that *TauT* is down-regulated by cisplatin in renal cells. Cisplatin represses the *TauT* gene at the transcriptional level through a p53-dependent manner, at least in part. Studies have shown that *TauT* is down-regulated by the p53 tumor suppressor gene in renal cells (Han *et al.*, 2002), and overexpression of p53 results in progressive renal failure in p53 transgenic mice, which is similar to observations made in taurine-deficient kittens (Godley *et al.*, 1996; Han *et al.*, 2000). The present study shows that forced overexpression of *TauT* can attenuate cisplatin-induced apoptosis of renal proximal tubule LLC-PK1 cells. Regarding the actions of p53 on *TauT* regulation and the role of *TauT* in renal development (Heller-Stilb *et al.*, 2002), we concluded that *TauT* may play an important role in protecting against drug-induced renal injury.

5. ACKNOWLEDGMENTS

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GENE EXPRESSIONS OF TAURINE TRANSPORTER AND TAURINE BIOSYNTHETIC ENZYME DURING MOUSE AND CHICKEN EMBRYONIC DEVELOPMENT

Ha Won Kim,[1] Seung Hyun Yoon,[1] Taesun Park,[2] Byong Kak Kim,[3] Kun Koo Park,[4] and Dong Hee Lee[1]

1. INTRODUCTION

Taurine ($^{+}NH_3CH_2CH_2SO_3^{-}$) is an abundant and widely distributed sulfur-containing amino acid that has been reported to be involved in diverse functions including modulation of calcium fluxes, membrane stabilization, maintenance of photoreceptor cells, modulation of neuronal excitability, antioxidation, osmoregulation, cell proliferation (Huxtable, 1992) and immune system (Fukuda *et al*., 1982; Green *et al*., 1991; Park *et al.*, 2002; Sapronov *et al.*, 2001). It has also been reported to improve brain functions in infants since taurine appears to play vital roles in the differentiation of neural stem cells (Benson and Masor, 1994). Other studies have also demonstrated that a critical level of taurine is essential for brain development and function, and that it plays important roles in various pathophysiologic states, such as epilepsy, migraine and brain ischemia (Dascal *et al*., 1987; Chesney *et al*., 1990; Domanske-Janik and Zablocka, 1993; Han *et al*., 1999). Taurine deficiency causes abnormal development of brain, retina, kidney, and abnormal function of the myocardium, where taurine plays an important role in intracellular calcium homeostasis in cardiomyocyte (Chesney *et al*., 1983, 1985).

Cysteinesulfinate decarboxylase (CSD) catalyzes the conversion of cysteine sulfinic acid (CSA) into hypotaurine. It can also transform cysteic acid (CA) to taurine (Jacobsen and Smith, 1968). CSD is involved in the biosynthetic pathway that leads from cysteine to taurine and is thought to be the rate-limiting step in taurine biosynthesis (De la Rosa and Stipanuk, 1985). CSD may thus be the key enzyme in the synthesis of taurine. Taurine is essential in fetal nutrition and development. However, the capability for endogenous biosynthesis of taurine is extremely low in the developing fetus of many animal

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Taurine 6
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systems, including humans. The enzyme activities of taurine biosynthesis are limited in early stage of embryonic development (Kuo and Stipanuk, 1984; Ghisolfi, 1987). As trans-placental transfer from the maternal origin is the primary source of taurine in the fetus, the taurine transporter is considered important along taurine biosynthetic enzyme in the fetus.

Taurine transporter (TauT), which contains 12 hydrophobic membrane-spanning domains, has been cloned in several species and tissues, including Madin-Darby canine kidney (MDCK) cells (Uchida *et al*., 1992), rat brain (Smith *et al*., 1992), mouse brain (Liu *et al*., 1992), human thyroid cells (Jhiang *et al*., 1993), human placenta (Ramamoorthy *et al*., 1994), pig kidney cells (Han *et al*., 1998; Han *et al*, 1999) and carp cells (Takeuchi *et al*., 2000). TauT belongs to the Na^+- and Cl^--dependent transporter gene family (Uchida *et al*., 1992) and its molecular mass is about 70 kDa (Miyamoto *et al*., 1995). Three potential N-glycosylation sites are conserved in the TauTs in the man, dog and rat. TauT activity is specifically down-regulated by PKC activation (Loo *et al*., 1996) and Ser-322 of the potential PKC phosphorylation sites is a critical site in the amino acid sequence defined in cloned TauTs (Han *et al*., 1999).

As taurine is especially essential to the fetus and newborn for their development, both biosynthesis and transportation of the taurine into the fetus are important during embryonic development. Thus, the gene expression of TauT and CSD using RT-PCR in mouse and chicken embryos was investigated to identify the expression phase during embryonic development.

2. METHODS AND MATERIALS

2.1. Cell Culture and RNA Isolation

Total RNAs of the ICR mouse embryo between days 4.5 and 18.5 were purchased from Seegene (Korea). Chicken eggs (white leghorn chicken; *Gallus domesticus*) were obtained from Jin Sung hatchery, Korea. The eggs were kept at 4°C until RNA was extracted from the organs of embryo. RAW264.7 cell line was obtained from American Type Culture Collection (Rockville, MD). The cell line was maintained in Dulbecco`s minimum essential medium (DMEM; GibcoBRL, France) supplemented with 10% heat inactivated fetal bovine serum (JRH Biosciences Co., Lenexa, USA) and penicillin (100 μg/ml) and streptomycin (100 μg/ml) (Life Technologies, Inc.) at 37°C in 5% CO_2. Total RNA was extracted from the cultured RAW264.7 cells using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. The amount of RNA was determined from the optical density at 260 nm, and the purity of RNA ascertained from the optical density ratio at 260 and 280 nm. RNA integrity was checked by electrophoresis on an agarose gel.

2.2. RT-PCR Amplification of mRNAs

Total RNA (2 μg) was denatured for 10 min at 70°C and mRNAs were reverse-transcribed (1 h, 42°C) in final volume of 20 μl containing reaction buffer (50 mM Tris-

Table 1. Primers for TauT and CSD in embryonic development stage

cDNA	Accession No (NCBI)	Primer	Sequence (5'→ 3')	PCR product (bp)	Anneal. (°C)
TauT	Z18956	F	TCGCGGATCCATACCGTATTTTA TTTTCCTGTTT	732	59
TauT	Z18956	R	TCTAGAATTCCCTGTACGAGTTA TACTTGTACTT	732	59
mCSD	AY033912	F	CTGCTTTTCTGGGACTTGGCACC	627	57
mCSD	AY033912	R	GGCTCCATGACCAACACAAATC	627	57
cCSD[a]	AY033912	F	TGTCCTGGTGGTTCCATCTCTAA	665	57
cCSD	AY033912	R	TTCCACATGAGCCACAGCTTC	665	57
mGAPDH	NM_008084	F	AGCCTCGTCCCGTAGACAAA	302	59
mGAPDH	NM_008084	R	CACGACATACTCAGCACCGGC	302	59
cGAPDH	AF047874	F	CCAACCCCCAATGTCTCTGT	401	59
cGAPDH	AF047874	R	CCTCTCACTGCAGGATGCAG	401	59

[a] Due to the limited information on chicken CSD cDNA sequence, primer pair of CSD was chosen in highly homologous parts of human (NM_015989), mouse (AY033912) and rat (NM_021750) CSD mRNA. F: forward, R: reverse. mCSD mouse CSD, cCSD: chicken CSD, mGAPDH mouse GAPDH, cGAPDH: chicken GAPDH.

HCl, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, pH 8.3), 0.5 μg/μl oligo(dT)12-18mer (Promega, USA), 1 mM deoxynucleotide triphosphate mixture (Biotools, Spain) and 200 unit M-MLV-RT (Moloney murine leukemia virus reverse transcriptase, Promega). After the reverse transcription, 2 μl of the RT products was amplified by PCR in final volume 25 μl containing PCR buffer (75 mM Tris HCl, 2 mM $MgCl_2$, 50 mM KCl, 20 mM $(NH_4)_2SO_4$, pH 9.0), 0.2 mM each deoxynucleotide triphosphate, and 1.25 units of *Taq* DNA polymerase (Biotools, Spain). PCR amplification was carried out using a thermocycler (Perkin Elmer, USA). The PCR program consisted of 30 cycles of 30 s at 94°C for denaturation, 30 s at 60°C for annealing, and finally 1 min at 72°C for extension. The PCR products were identified by 1% agarose gel electrophoresis. The primers were designed using Primer Express software version 1.0 (PE Applied Biosystems, USA) by using published RNA sequences from GenBank (Table 1). Primers for real-time PCR were limited to amplify products of 200 bases or less. Primers were ordered from DyneBio (Takara, Korea).

3. RESULTS

3.1. Expression of TauT and CSD mRNAs in Mouse Embryo

To analyze the expression phases of TauT and CSD during embryonic development, the gene expressions of TauT and CSD were analyzed using RT-PCR in mouse embryos. Total RNAs of ICR mouse embryo at days 4.5, 10.5, 15.5 and 18.5 were purchased from Seegene (Korea). Primers for TauT and CSD used were shown in Table. 1. RT-PCR analysis revealed that both TauT and CSD mRNAs were already expressed at day-4.5 in mouse embryo (Fig. 1). However, mouse pure embryo total RNA at day 4.5 was technically difficult to obtain and thus contaminated with extra-embryonic tissues such as maternal uterus. As TauT is reported to be expressed abundantly in placenta (Ramamoorthy *et al.*, 1994), we changed to chicken embryo system to obtain pure embryo mRNA.

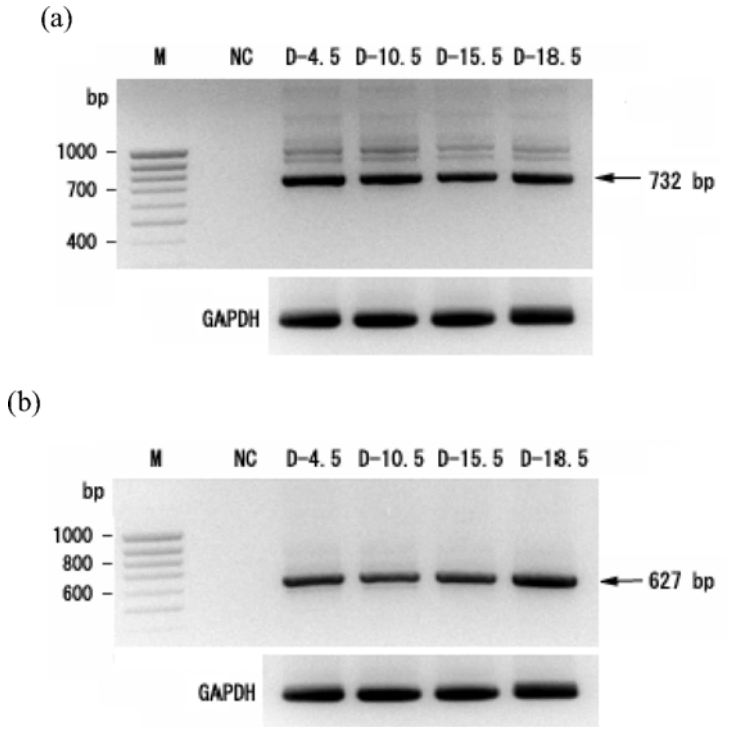

Figure 1. Expression of TauT and CSD mRNAs in mouse embryo development. RT-PCR analysis of 2 total RNA from mouse embryos at age 4.5, 10.5, 15.5 and 18.5 days using primers specific to TauT (a) and CSD (b). Lane NC represents the absence of cDNA, and GAPDH was used as a control to confirm equal addition of mRNA in PCR reactions.

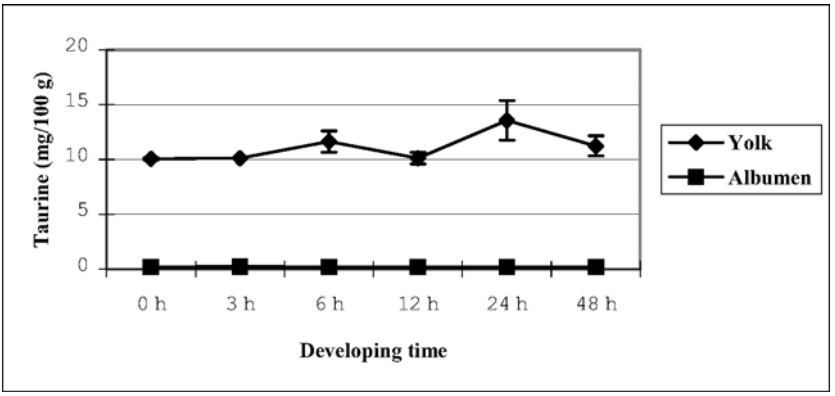

Figure 2. Concentration of taurine in chicken egg yolk and albumen. Taurine contents were determined by HPLC equipped with RF-detector. The yolk and albumen were diluted by 20% and 40% for analysis, respectively. Each value represents the mean ± SEM (n=3).

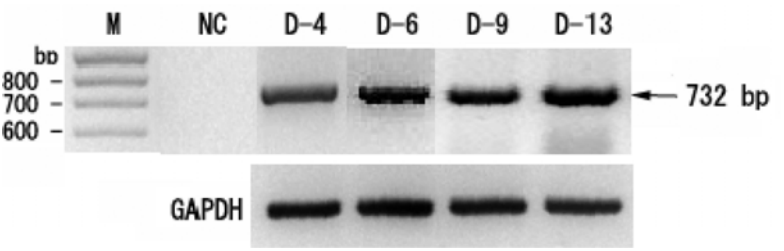

Figure 3. Expression of TauT mRNA during chicken embryonic brain development. RT-PCR analysis of 2 μg total RNA from 4-, 6-, 9- and 13-day-old chicken embryo brains using primers specific to TauT. Lane NC represents the absence of cDNA, and GAPDH was used as a control to confirm equal addition of mRNA in PCR reactions.

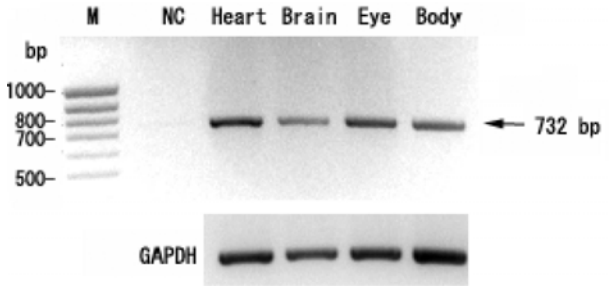

Figure 4. Expression of TauT mRNA in the organs of chicken embryos at day 3. RT-PCR analysis of total RNA from chicken embryo at the age of 3 days using primers specific to TauT. Lane NC represents the absence of cDNA, and GAPDH was used as a control to confirm equal addition of mRNA in PCR reactions.

3.2. Expression of TauT mRNA During Chicken Embryo Development

Prior to analyzing the gene expression of TauT and CSD in chicken embryo, taurine concentrations in chicken eggs were measured by HPLC. The yolk and albumens were collected and analyzed from the eggs of 0, 3, 6, 12, 24 and 48 h of developing times. As shown in Fig. 2, taurine mostly existed only in the yolk but not in albumen. The estimated concentration of taurine in the yolk was between 10.1 and 13.6 mg/100 g.

After isolating the embryos from eggs at days 4, 6, 9 and 13 of development, the expression phases of TauT and CSD during chicken embryonic development were analyzed. As taurine is reported to be expressed in the brain, we tried to analyze TauT expression in the chichen embryo brain. As shown in Fig. 3, TauT mRNAs were highly expressed in all embryo brains at days 4, 6, 9 and 13 by the analysis of RT-PCR. Then, we decide to analyze TauT expression in various organs of chicken at day 3. TauT was also expressed in the organs of heart, brain, eye and rest of head in chicken embryos as early as day 3 (Fig. 4). Among the organs, TauT was strongly expressed in the heart and eye and weakly in the brain at day 3 chicken embryo. Therefore, these observations were extended to developing chicken embryos earlier than day 3.

3.3. Gene Expression of Taurine Biosynthetic Enzyme Precedes by TauT

We decided to analyze TauT and CSD expressions earlier than at day 3 in the chicken embryo. When we analyzed mRNA expressions by RT-PCR, TauT and CSD were detected in the chicken embryo as early as day 2 (Fig. 5a). The gene of CSD was expressed as early as at 12 h, however, the expression of TauT mRNA was not expressed even at 24 h in chicken whole embryo (Figs. 5b and c). Our data show that TauT and CSD mNAs began to appear at the developing times of 48 h and 12 h, respectively. The taurine synthetic enzyme CSD was expressed first and only then the TauT gene in the developing chicken embryo. Therefore, the chicken embryo is supplied taurine in part from the egg yolk and in part from the synthesis in the embryo.

4. DISCUSSION

Taurine is required for a number of biological processes. It is especially essential to the fetus and newborn for their development. Taurine is necessary for human fetal brain neuron proliferation and differentiation (Chen *et al.*, 1998). Taurine is regarded as a putative human fetal brain neurotrophic factor in the process of human brain development, promoting both proliferation and differentiation of brain cells (Chen *et al.*, 1998). The fetus and newborn, however, have little biosynthetic capacity (Ghisolfi, 1987). The nutritional and physiological requirements for taurine in mammals are partly met by dietary sources and partly by biosynthesis. To identify the expression phase of TauT and biosynthetic enzymes of taurine CSD during embryonic development, their gene expression was analyzed using RT-PCR in mouse and chicken embryos. The results showed that both TauT and CSD mRNAs were already expressed at day 4.5 in the mouse embryo (Fig. 1). In the chicken whole embryo, TauT and CSD mRNAs began to appear on developing times of 48 h and 12 h, respectively (Fig. 5).

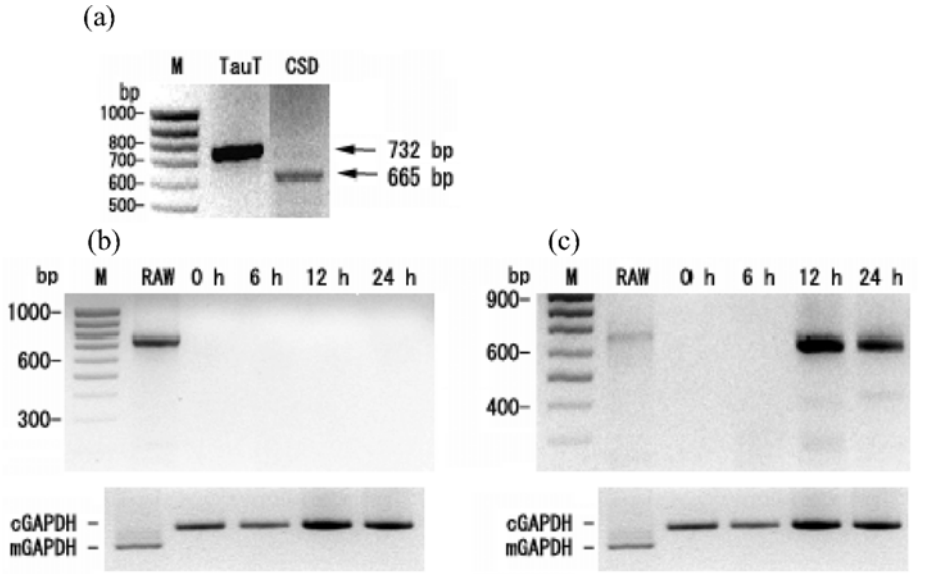

Figure 5. Expressions of TauT and CSD mRNAs in the chicken whole embryo development. RT-PCR analysis of 2 total RNA from chicken embryo at day 2 using primers specific to TauT and CSD (a). Expression of TauT (b) and CSD (c) mRNA from chicken embryos at developing times of 0 h, 6 h, 12 h and 24 h. cGAPDH: chicken GAPDH; mGAPDH: mouse GAPDH.

In case of transporter gene expression in the embryo such as glucose transporter, each expression of isoforms of glucose transporter (Glut1-5 and 8) was reported at different developmental stages in bovine embryos (Augustin *et al*., 2001). Although Glut1, Glut3 and Glut8 were expressed at all stages studied, Glut4 and Glut5 expressions were first detected in the blastocyst and 8-/16- cell stages, respectively. Glut2 expression was restricted to the period of blastocyst elongation at day 14 and day 16 (Augustin *et al*., 2001). Another study also reported that the copper transporter CTR1 was expressed at age 6.5 days of developmental stages and was essential for embryonic growth and development (Kuo *et al*, 2001).

In our embryonic gene expression study on the mouse embryo it was impossible to study early gene expression due to the difficulty of separation of the fetus from placental tissues. Upon preparation of fetal embryo tissue from the day 4.5 placenta, the fetus was inevitably contaminated with placental tissues when the fetus was detached from the placenta. Therefore, we used the chicken embryo system for the analysis of early gene expression of TauT and CSD genes. Even in the day 3 chicken embryo, TauT gene was expressed in various organs such as the heart, eye, brain and other tissues as shown in Fig. 4. As a high concentration of taurine was present in the egg yolk, the fetus could be supplied taurine from the egg yolk. As TauT began to be expressed from day 2, the embryo can utilize taurine only after day 2. Taken together, TauT and CSD mRNAs were expressed in early stages of embryonic development, indicating important roles of taurine in the developing fetus. Further studies are needed for gene expression of these enzymes in case of mammalian embryos such as mouse and human.

5. CONCLUSIONS

Taurine (2-aminoethanesulfonic acid) is one of the major intracellular β-amino acids in mammals and is required for a number of biological processes including membrane stabilization, osmoregulation, modulation of calcium flux, antioxidation, neuromodulation, cell proliferation, and immune systems. As taurine is essential to the fetus and newborn for their development, the transfer of taurine into fetus is important during embryonic development. However, the protein expression pattern of taurine biosynthesis and transportation in the early embryonic development stage is still unknown. Thus, we have investigated the gene expression of TauT and CSD, which is one of the rate-limiting enzymes of taurine biosynthesis, using reverse transcriptase-polymerase chain reaction (RT-PCR) in mouse and chicken embryos to identify the expression phase during embryonic development. Murine embryos aged 4.5 days expressed both mRNAs of TauT and CSD. To overcome difficulties in the analyses of very immature embryos, the chicken embryo was employed instead of murine one. The chicken embryo aged 3 days produced TauT mRNA in the heart, brain and eye. In the analyses on chicken whole embryos, mRNAs of CSD and TauT began to appear at 12 and 48 h, respectively. These data show that TauT and CSD mRNAs are expressed in early stage of embryonic development and taurine synthetic enzyme is expressed earlier than that of the TauT, indicating important roles of taurine in the developing fetus.

6. ACKNOWLEDGMENTS

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MECHANISMS OF REGULATION OF TAURINE TRANSPORTER ACTIVITY

A Complex Interplay of Regulatory Systems

Xiaobin Han and Russell W. Chesney*

1. INTRODUCTION

In mammals, the taurine total body pool is regulated at the site of renal proximal tubule, where taurine is transported by a transmembrane protein – the taurine transporter (Friedman *et al.*, 1981; Chesney *et al.*, 1985). The original studies showed that renal tubular epithelium can adapt to alterations in the sulfur amino acid composition of the diet - the phenomenon was described as renal adaptive regulation (Friedman *et al.*, 1983; Chesney *et al.*, 1985), and has been found in man, pig, dog, rat, mouse, and cat (Chesney *et al.*, 1986). Taurine transport is also modulated by protein kinase C (PKC). Activation of PKC by active phorbol 12-myristate 13-acetate (PMA) reduces NaCl-dependent taurine uptake by LLC-PK1 cells (of porcine proximal tubular origin) (Jones *et al.*, 1991). Recently, cloning of the taurine transporter gene (*TauT*) and *TauT* promoter provides an enormous opportunity for us to explore the mechanisms of how the *TauT* gene and its products are regulated *in vivo*. The findings from these studies have begun to unveil the physiological significance of the adaptive regulation of *TauT* in man and other mammalian species.

2. MATERIALS AND METHODS

2.1. Construction of the Reporter Gene

The promoter region of *TauT* was identified in previous studies (Han *et al.*, 2000a). Binding site(s) for transcription factors, including WT1-, p53-, c-myb-, and c-Jun were found in the *TauT* promoter sequence. In this study, approximately 1.1 kb of the *TauT*

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Taurine 6
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promoter region DNA (GenBank accession number AR151716) was used as the template for PCR and the PCR fragment was cloned into the promoterless luciferase vector pGL3-Basic (Promega, Madison, WI) to generate the plasmid p963 for use in transfections and luciferase assays. The conditions used are 30 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 58°C, and 1 min of elongation at 72°C. The sense primer (5'-GGGGTACCTTACTGAAGGTCACACAGC-3') designed for PCR contained a unique site for *KpnI*, and the antisense primer (5'-AAGATCTTGGCACGGGAGTTCA-3') contained a unique site for *BgI II*. PCR products were digested with *KpnI* and *BgI II* and re-ligated into *KpnI* and *BgI II* sites of pGL3-Basic to generate plasmids containing segments of the *TauT* promoter sequence extending from the +48 nucleotide corresponding to the transcriptional start site. The constructs were verified by DNA sequencing. The 5'-progressive deletions were generated from the p963 plasmid by using sense primers 5'-GGTTCTTTGTGTGTCCGAGCTCCTG-3'(p265), 5'-GGGGTACC-TGTGTGTGGGCGT-3'(p182), 5'-GGGGTACCAGACCCCGCCCTA-3' (p-sp1), 5'-GGGGTACCTAGGCCCCGCCCA-3'(p-sp2), 5'-GGGGTACCAGGCCCGGCCAAG-3'(p-sp3), and 5'-GGGGTACCAGCAGGATGGGTG-3'(p-sp4), respectively. The antisense primer used for these constructs was the same as described above. The 3'-progressive deletion were generated from p963 by using the same sense primer for p182 and antisense primers 5'-GGGGCCTGGGAGGTCAGCCACG-3' (pd-sp1), 5'-GGGGCCTGGGGAGGGGTCTGGGCGGT-3'(pd-sp2), 5'-CCGGGCCTGGGAGGGG-CCTA-3'(pd-sp3), and 5'-CCGGGCCTGGGCGGGGCCTA-3'(pd-sp4).

2.2. Cell Culture

LLC-PK1, Rat1a, NKR-52E, (10)1, and 293 (human embryonic kidney) cells were cultured according to ATCC (American Type Culture Collection) guidelines. Briefly, cells were grown as confluent monolayers in 10 cm diameter tissue culture plates in media specific for each cell line with 10% fetal calf serum at 37°C in the presence of 5% CO_2 in a humidified incubator. Cells were plated 18 h before transfection and fed with fresh medium 4 h before transfection.

2.3. Transient Transfection

Plasmid DNA was introduced into cultured mammalian cells using cationic liposomes (Lipofect AMINE, Life Technologies, Grand Island, NY). Transfection was carried out for 16-18 h, and then cells were washed twice with phosphate-buffered saline and incubated in fresh medium for 24-48 h before harvesting. pGL-control, which contains a luciferase gene driven by the SV40 early region promoter/enhancer, and empty pGL-Basic vectors were used as positive and negative controls, respectively. To standardize the transfection efficiency, 0.1 µg of pRL-CMV vector (pRL Renilla Luciferase control reporter vector, Promega, Madison, WI) was cotransfected in all experiments. Transfected cells were harvested 48 h after transfection and lysed in 200 µl of reporter lysis buffer (Promega, Madison, WI)). A luciferase assay was performed using a dual-luciferase assay kit (Promega, Madison, WI), and activity was measured with an Optocomp 1 luminometer (MGM Instruments, Inc., Hamden, CT). Promoter activity (mean ± S.D. of four samples in relative light units) of each construct is represented by relative light output normalized to pRL-CMV control. Graphs represent typical results of four separate experiments. The

concentration of protein in the cell extracts was determined using the Bradford method (Bio-Rad, Hercules, CA).

2.4. Site-Directed Mutagenesis and *in vitro* RNA Synthesis

An *in vitro* mutagenesis system (QuikChange, Stratagene) was used for site-directed mutagenesis using the MDCK taurine transporter (pNCT) cDNA as a template. Mutations were confirmed by enzymatic nucleotide sequencing (United States Biochemical Corp). Capped RNA transcripts were synthesized *in vitro* with SP6 RNA polymerase following the manufacturer's instructions (mCAP RNA Capping Kit, Stratagene).

2.5. Microinjection of *Xenopus Laevis* Oocytes

Ovarian lobes were dissected from anesthetized frogs and oocytes were separated by incubation of ovarian fragments for 40 minutes with 2 mg/ml collagenase type II in calcium-free buffer at room temperature. Defolliculated stage V-VI oocytes were selected and incubated overnight at 18ºC in media (50% L-15, Leibovitz, Sigma), 1 mM L-glutamine, 15 mM HEPES, pH 7.6, 100 μg/ml gentamicin sulfate) before injection. Water (30 nl) containing 30 ng of capped transcripts was injected into each oocyte. Injected oocytes were maintained at 18ºC in the above media for 3 days prior to taurine uptake experiments.

2.6. Measurement of Taurine Uptake

Oocytes were transferred to a Na^+-containing uptake solution referred to as ND96 (2 mM KCl, 1 mM $MgCl_2$, 96 mM NaCl, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.6) to which was added 10 μM unlabelled taurine and 0.5 mCi/l [^{14}C]taurine (Du Pont, Perkin-Elmer). After incubation for 30 min at room temperature, oocytes were transferred to a 24-well cell culture plate in which they were washed rapidly 5 times with 1 ml of ice-cold Na^+-free uptake solution. Lastly, individual oocytes were transferred to miniscintillation vials, solubilized in 100 μl of 10% SDS, and counted in 2 ml of scintillation cocktail (Aquasol, NEN).

2.7. Statistics

All experiments were performed in triplicate. Luciferase assays are expressed in units of relative light output. The data represent the mean ± SEM of 3 or 4 experiments. Statistical comparisons were made using one-way ANOVA and Student's t test to determine significant differences in the means.

3. RESULTS

3.1. Adaptive Regulation of Brush Border Membrane Vesicle (BBMV) Taurine Transporter Activity by Dietary Taurine

To study whether dietary changes affect the accumulation of taurine by BBMV, taurine uptake by BBMV of the rats fed each diet was measured. As shown in Fig. 1, the

accumulation of taurine is greater by BBMV prepared from rats fed the LTD and lower in BBMV from HTD-fed animals, suggesting that expression of *TauT* is adaptively regulated by dietary manipulation.

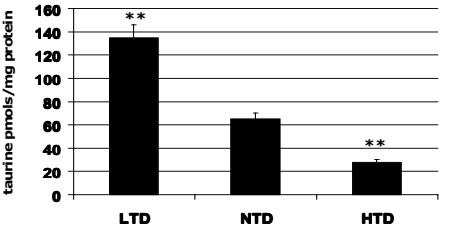


Figure 1. Effect of diet on brush border membrane vesicle uptake of taurine. Sprague-Dawley rats of age 12 weeks were fed LTD, NTD or HTD for 14 days, and then taurine uptake by BBMV was measured. Data represents mean ± SD from three rats in each group. ** $p<0.05$ *vs*. NTD.

3.2. Effect of Diet on Urinary Taurine Excretion

To determine the effect of diet on urinary taurine excretion, rats were fed with low taurine diet (LTD), normal taurine diet (NTD) or high taurine diet (HTD) for 14 days, and then clearance of taurine was measured. As shown in Fig. 2, urinary taurine is significantly lower in LTD-fed rats and higher in HTD-fed rats than in NTD animals.

3.3. *TauT* is Adaptively Regulated by Dietary Taurine Through TREE

Studies have shown that expression of *TauT* is transcriptionally regulated by dietary taurine through the Ca^{2+} signal pathway (Han *et al.*, 1997, 2000), suggesting that a taurine response element (TREE) exists in the *TauT* promoter region. To identify the TREE, we have generated several reporter genes by series deletion of p963 and adaptive regulation of these reporter genes by taurine was determined in LLC-PK1 cells. As shown in Fig. 3, the adaptive regulation was observed in p963, but not in p574 and p265, suggesting that the TREE is located between -963 and -574.

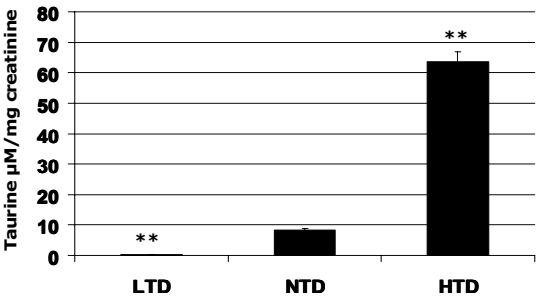


Figure 2. Effect of diet on urinary taurine level. Sprague-Dawley rats of age 12 weeks were fed LTD, NTD or HTD for 14 days, and then urinary taurine level was measured. Data represent mean ± SD of taurine (µmol/mg creatinine) from three rats in each group. ** $p<0.01$ *vs*. NTD.

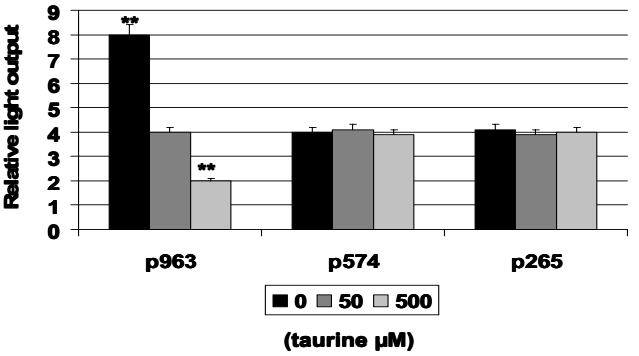


Figure 3. TREE is required for renal adaptive regulation of *TauT*. Reporter genes were transiently transfected into 293 cells cultured in medium containing 0, 50, or 500 μM taurine for 24 hours, then a luciferase assay was performed with the cell lysates. Promoter activity is represented by relative light output normalized to pRL-CMV control. The graph represents typical results of four separate experiments. ** $p<0.05$ *vs*. control (50 μM taurine).

3.4. Ser-322 Is a Critical Site for PKC Regulation of Taurine Transporter

Several potential PKC sites are found in TauT, including Ser-45, Thr-175, Ser-215, Thr-242, Ser-322, and Thr-581. Each of these residues was replaced by alanine using site-directed mutagenesis. The mutants were designated S45A, T175A, S215A, T242A, S322A, and T582A, and then expressed in oocytes. Taurine transport activity by the S322A mutant was increased more than three-fold compared with wild-type TauT, whereas the other mutant transporters were not different from the wild-type TauT. Activation of PKC by PMA (100 nM) downregulates taurine transport activity by wild-type taurine transporter and all mutants, except Ser-322 (Fig. 4).

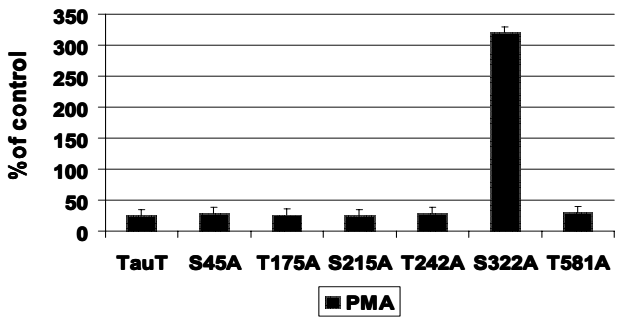


Figure 4. Effects of PKC activation on taurine uptake. Wild-type or mutant TauT-expressing oocytes were incubated with PMA for 30 min, then taurine uptake was measured. Taurine uptake in wt-TauT-injected oocytes was used as a control. Data for each condition are given as percentages of control and are representative of three experiments. Each value represents the mean ± SEM for six oocytes.

3.5. S4 Segment of TauT is Involved in the Gating of Taurine

To determine the role of the charged residues in S4, we individually mutated each of these residues in TauT. We then compared the function of the mutants with that of the wild-type transporter by expressing the transporters in *Xenopus laevis* oocytes. As shown in Fig. 5, taurine uptake by oocytes expressing mutant K319Q (lysine-319 replaced by glutamine) and D325Y (aspartic acid-325 replaced by tyrosine) was increased by about 50% compared with the control (wild-type TauT). In contrast, when arginine-324 was replaced by glycine (mutant R324G), taurine transport was decreased by 70% compared with the control, while replacing lysine with glutamine (mutant D317Q) had no effect on taurine transport activity.

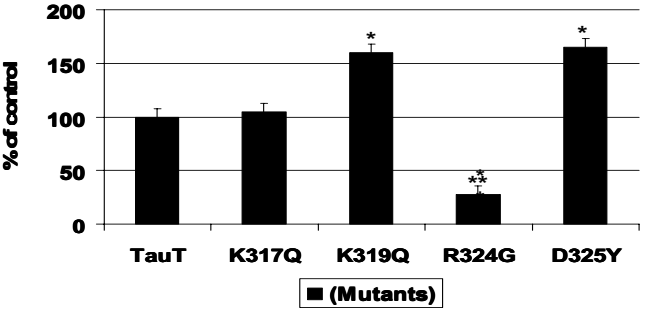


Figure 5. Effect of mutation of charged residues of Taut on taurine uptake. Oocytes were injected with 30 ng of wt-TauT or mutant cRNA and assayed for taurine uptake 3 days post-injection. Data for each mutant are given as percentages of the control and are representative of three experiments. Each value represents the mean ± SEM for six oocytes. * $p<0.05$ *vs*. control, ** $p<0.01$ *vs*. control.

3.6. *TauT* is Transcriptionally Repressed by p53 in Renal Cells

To determine if down-regulation of the *TauT* gene by p53 occurs at the transcriptional level, the reporter construct pGL-963, containing a putative p53-binding site from -663 to -695 (Fig.6), was transiently transfected into 293, NRK-52E, and (10)1 cells. Regulation of *TauT* promoter function by p53 was examined in cells treated with or without doxorubicin. As shown in Fig. 6, doxorubicin decreased TauT promoter activity in both 293 and NRK-52E cells, but had no effect in the (10)1 cells.

3.7. *TauT* Is Transcriptionally Activated by WT1

To determine if up-regulation of the *TauT* gene by WT1 occurs at the transcriptional level, several constructs (including p265, which contains a putative WT1/EGR-1/Sp1-binding site from -160 to -171) were created by progressive deletion and were transiently transfected into 293 cells. Regulation of *TauT* promoter activity by WT1 was examined. As shown in Fig. 7, reporter gene p265, which contains a TG repeat, shows strong promoter activity that was enhanced by WT1. Deletion of the TG repeat (p182) repressed *TauT* promoter activity and its up-regulation by WT1. Deletion of the WT1/EGR-1/Sp1 site (dpWT) abolished the effect of WT1 on *TauT* promoter function. Consistent with a

previous study, we demonstrated that an Sp1 site is essential for the basal promoter function of *TauT* in 293 cells.

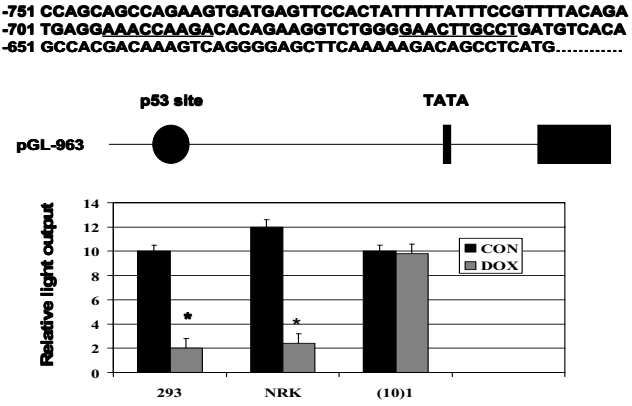


Figure 6. Down-regulation of *TauT* promoter activity by p53 in renal cells. A consensus p53-binding site located at -663 to -695 relative to the transcription start site is shown. pGL-963 was transiently expressed in 293, NRK-52E cells treated with or without doxorubicin (Dox) for 48 h. The luciferase assay was performed in the cell lysates. The graph represents typical results of four separate experiments. * P<0.01 *vs*. control cells.

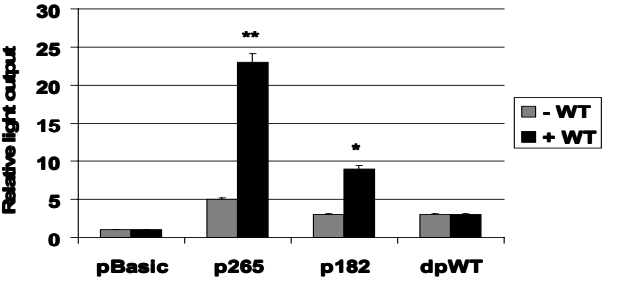


Figure 7. WT1/EGR-1/Sp1 consensus site is for WT1 regulation of *TauT* promoter activity. The effect of deletion of the WT1 site on activation of *TauT* promoter by WT1 was studied in 293 cells. Reporter gene constructs were transiently transfected into 293 cells for 24 h, and then a luciferase assay was performed, as described in the Methods. The promoter activity (mean ± S.D. of four samples) is represented by relative light output normalized to pRL-CMV control. The graph represents typical results of four separate experiments.

3.8. *TauT* is a Target Gene of c-Jun

Two AP1 binding sites have been found in the *TauT* promoter located at -367 to -374 and -642 to -648. To determine whether *TauT* is regulated by c-Jun, the reporter gene constructs containing two AP1 sites or AP1 site mutations were co-transfected with c-Jun into Ratla cells, and transcriptional regulation of *TauT* by c-Jun was studied. As shown in Fig. 8, promoter activity of *TauT* is up-regulated by c-Jun (p963). Mutation of a single AP1 site decreases the effect of c-Jun on *TauT* promoter [p963/KS (-367 to -374) and

p963/AS (-542 to -548)], while mutation of both AP1 sites result in the loss of the activation induced by c-Jun (p963/AA).

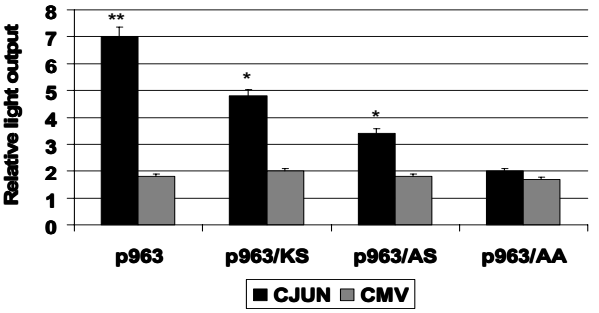


Figure 8. Regulation of *TauT* promoter by c-Jun oncogene. Reporter genes (p963), p963/KS, p963/AS, and p963/AA were co-transfected with c-Jun (black) or CMV (grey) into Ratla cells. The promoter activity (mean ± S.D. of four samples) is represented by relative light output normalized to pRL-CMV control. The graph represents typical results of four separate experiments. * $p<0.05$ vs control, ** $p<0.01$ vs control.

3.9. *TauT* Appears to be a Responsive Gene of c-Myb Proto-oncogene

A c-Myb binding site has been located between the p53 binding site and the Ap1 binding site. To determine whether transcription of the *TauT* gene is regulated by c-Myb, a pGL-963 reporter gene containing the c-Myb consensus site(s) was co-transfected with *c-myb* cDNA into MCF-7 cells for 24 h. As shown in Fig. 9, the promoter activity of the *TauT* gene was increased more than 20-fold by c-Myb, and deletion of the consensus site(s) for c-Myb abolished the effect, suggesting that *TauT* may be a target gene of c-Myb.

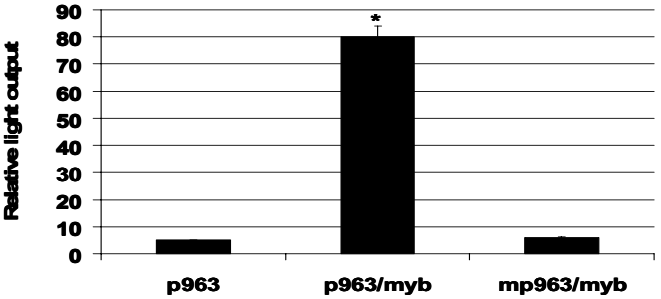


Figure 9. Effect of c-Myb on the promoter activity of *Tau**T***. pGL-963 or a mutant pGL-963 was transiently transfected into MCF-7 cells for 24 h. A luciferase assay was performed with the cell lysates. The promoter activity was represented by relative light output normalized to pRL-CMV control. The graph represents typical results of four separate experiments.

4. DISCUSSION

Renal adaptive regulation of the taurine transporter gene (*TauT*) which occurs at the S3 segment of renal proximal tubule, has been well studied during the past two decades. Recent studies have shown that taurine and taurine transporter play an important role in retinal, brain and kidney development (Han *et al.*, 2000b; Heller-Stilb *et al.*, 2002). Therefore, study the mechanisms of how the *TauT* and it products are regulated could reveal the significance of renal adaptive regulation of *TauT* and its role in maintaining normal kidney functions.

We have shown that the S4 segment of the *TauT* transmembrane protein involves in the gating of taurine across cell membrane. Enhanced taurine uptake by K319Q, rather than K317Q, suggests that lysine-319 may play an important role in the formation of the amino acid consensus sequence for taurine transport. Furthermore, the positively charged lysine-319 and negatively charged aspartic acid-325 may attract one another and thus help to maintain the status of the loop, which is important for controlling the gating function of the taurine transporter, Arginine-324 may function as a binding site for taurine, because kinetic analysis of mutant R324G demonstrated that decreased taurine transport was the result of an increase in the K_m of the transporter. Phosphorylation of Ser-322 by PKC could potentially change the 3-dimensional structure of the gate and block the ionic binding of taurine to Arg-324.

This study demonstrated that Ser-322 on the S4 segment of the transporter protein is the critical site for PKC phosphorylation, which modulates the function of the taurine transporter. Consistent with previous results using an antibody against S4, taurine uptake by oocytes expressing an S322A mutant was 3-fold higher than that of oocytes expressing the wild-type transporter. The S322A mutation also abolished the inhibitory effect of PMA on taurine transporter activity.

In another study we demonstrated that the *TauT* gene is transcriptionally regulated by Sp1, WT1, p53, c-Jun, and c-Myb. Sp1 is required for maintainance of the basal promoter activity of *TauT*, which in turn keeps *TauT* expression at a steady level. Up-regulation of *TauT* by WT1 is likely to be essential during renal development. Since WT1 plays a critical role in kidney development (Kreidberg *et al.*, 1993), and knockout of *TauT* shows an abnormal development of kidney (Heller-Stilb *et al.*, 2002).

p53 represses *TauT* expression by directly binding to the *TauT* promoter region. Varmus' group found that transgenic mice over-expressing p53 undergo progressive renal failure through a novel mechanism by which p53 appeared to alter cellular differentiation, rather than by growth arrest or the direct induction of apoptosis (Godley *et al.*, 1996). These findings suggest that altered expression of certain p53 target gene(s) that are involved in renal development may be responsible for p53-induced progressive renal failure in p53 transgenic mice. Interestingly, the progressive renal failure found in p53 transgenic mice is similar to observations in the offspring of taurine-deficient cats that showed ongoing kidney damage in addition to abnormal renal and retinal development (Han *et al.*, 2000b), suggesting that the taurine transporter gene is an important target of p53.

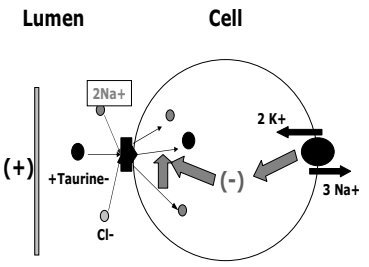
Several lines of evidence suggest that the interaction of p53 and WT1 plays an important role in normal development and in tumorigenesis. Using *in vitro* immunoprecipitation and Western blot analyses, p53 and WT1 proteins were shown to physically interact in BRK (baby rat kidney) cells (Maheswaran *et al.*, 1993). WT1

protein was shown to stabilize p53, modulate its transactivational properties, and inhibit its ability to induce apoptosis (programmed cell death) (Maheswaran *et al.*, 1995).

c-Jun is a versatile transcription factor and contributes to transformation and tumor aggressiveness (Hartl *et al.*, 1995; Smith *et al.*, 1999) as well as cell cycle progression (Wisdom *et al.*, 1999), differentiation (Szabo *et al.*, 1991), and apoptosis (Bossy-Wetzel *et al.*, 1997) in a tissue and cell-specific manner. It acts in conjunction with Ras to transform primary cells (Alani *et al.*, 1991) and is essential for transformation of cells by Ras (Johnson *et al.*, 1996). In addition to being necessary for Ras transformation of cells, c-Jun is also required for transformation induced by c-fos, raf, c-myc, mos and abl (Rapp *et al.*, 1994). In this study we found that *TauT* is transcriptionally activated by c-Jun, suggesting that *TauT* may play a role in oncogene-induced cell transformation.

The *c-myb* product (c-Myb) is highly expressed in immature hematopoietic cells, and its expression is down-regulated during terminal differentiation. A similar phenomenon was also observed for *TauT* during hematopoietic cell differentiation (Learn *et al.*, 1990). We have found that the promoter region of *TauT* contains several consensus binding sites for c-Myb. In a recent study we demonstrated that *TauT* is upregulated by c-Myb, suggesting that *TauT*, as a potential new target gene of *c-myb*, may play a role in myeloid development.

Taken altogether, we summarize the taurine transporter system and the signaling pathway of *TauT* regulation as shown in Figs. 10 and 11.



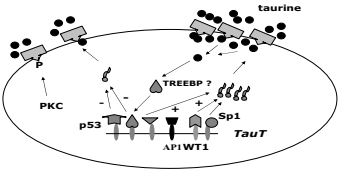


Figure 10. Model of $2Na^+$:Cl^-:taurine Transporter System **Figure 11.** Intracellular signaling of *TauT* regulation

In conclusion, *TauT* appears to be a development-related gene that is especially involved in renal development. It is supported by the fact that *TauT* is a target gene of WT1. Expression of *TauT* is primarily controlled by TREE in response to the availability of dietary taurine. This adaptive regulation may represent a role for *TauT* in maintaining the normal function of kidney. Expression of *TauT* is also regulated by transcription factors p53, WT1, Sp1, c-Jun, and c-Myb, suggesting that *TauT* may play a variety of roles in cell development, apoptosis, transformation, and tumorigenesis.

5. ACKNOWLEDGMENTS

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TauT GENE EXPRESSION IS REGULATED BY TonEBP AND PLAYS A ROLE IN CELL SURVIVAL

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1. INTRODUCTION

Taurine is stored at millimolar concentrations in all mammalian tissues and has several cytoprotective properties, such as calcium handling, osmoregulation, antioxidation, and detoxication (Huxtable, 1992; Satoh and Sperelakis, 1998; Schaffer *et al*., 2000). The tissue taurine level is determined by biosynthesis from methionine and cysteine and the uptake from plasma via taurine transporter (TauT). Intracellular taurine concentrations are extensively as high as 10 mmol/kg wet tissue in many mammalian tissues, while taurine is found at the concentrations of 20-100 μM in plasma (Chesney, 1985; Huxtable, 1992; Chapman *et al*., 1993), implying that TauT plays an important role in the maintenance of the high concentration of taurine in tissues.

When cells are exposed to hyperosmotic conditions, changes in the cell volume and ionic strength occur, which results in cell growth arrest and cell injury. On the other hand, mammalian cells adapt to the hyperosmotic environment by accumulating organic osmolytes, such as sorbitol, betaine, and myo-inositol, as well as taurine. This adaptive process involves changes of gene expression that lead synthesis or transport of osmolytes, including aldose reductase (AR), betaine/GABA transporter (BGT), sodium/myo-inositol transporter (SMIT) and TauT (Burg *et al*., 1997).

Transcriptional factor tonicity-responsive element (TonE) binding protein (TonEBP)/ nuclear factor of activated T-cell (NFAT5), a member of Rel family, plays a central role in protecting cells from the hypertonic stress (Woo *et al*., 2002; Ho, 2003). TonEBP is activated by hypertonicity and, in turn, stimulates the transcription of several osmoprotective genes, including SMIT, BGT-1, AR, heat shock protein 70 (Hsp70), and so on (Woo *et al.,* 2002; Ho, 2003). TonEBP is also expressed in several tissues which are not exposed to hypertonic stress, such as heart, skeletal muscle, brain, and embryonic

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Taurine 6
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stem cells (Miyakawa *et al*., 1999; Maouyo *et al*., 2002). It has been demonstrated that TonEBP-knockout mice displayed fetal lethality (Lopez-Rodriguez *et al*., 2004), suggesting that TonEBP possesses multiple functions in addition to osmoregulation. However, the physiological function in these tissues is unexplored.

Since TauT is also upregulated by hypertonic stress as an adaptive response similarly to the other osmoprotective genes, we hypothesized that TauT is also regulated under the control of TonE sequence in response to hypertonicity. To examine this hypothesis, we analyzed the *cis*-element responding to hypertonicity in 5'-flanking region of the *TauT* gene. Moreover, we investigated the role of TonEBP in the regulation of TauT expression in cardiomyocytes.

2. METHODS

2.1. Cell Culture and Experiments

HepG2 cells were exposed to osmotic stress by culturing in hypertonic media. Hypertonic media were prepared by adding 50 mM sodium chloride (NaCl +50) or by 100 mM sucrose (Suc 100).

Cardiomyocytes were isolated from the heart of 0-day-old neonatal Wister-Kyoto rat as described previously. In brief, cardiac ventricles were minced and dissociated with 0.1% trypsin (Invitrogen, Carlsbad, CA, USA) and 0.1% collagenase type IV (Sigma Aldrich, St. Louis, MO, USA). Dispersed cells were plated and incubated for 1 h at 37ºC. Non-attached myocytes were collected and cultured in Dulbecco's modified essential medium/Ham's F-12 (DMEM) containing 5% neonatal bovine serum, 0.1% penicillin/streptomycin and 100 μM bromodeoxyuridine (BrdU) for 2 days. After washing twice with serum-free DMEM, the cells were treated with doxorubicin in serum- and BrdU-free DMEM.

Total RNA and protein were isolated after culturing for 24 h and assessed to Northern blot or Western blot, as described previously (Ito *et al*., 2004).

2.2. Plasmids

Promoter-reporter constructs pTauT/-269-Luc, pTauT/-124-Luc, pTauT/-99-Luc, and pTauT/-124mut-Luc were generated as described previously (Ito *et al*., 2004). Briefly, each fragment containing the TauT promoter region were prepared by PCR using followed primers (Invitrogen) and cloned into firefly luciferase plasmid pGL3; -269 (forward): 5'- GGG GTA CCC GGG TTC TTT GTG -3', -124 (forward): 5'- GGG GTA CCC GGC CAA GCT GGT ATT -3', -99 (forward): 5'- GGG GTA CCC AGC AGG ATG GGT GAT -3', -124m (forward): 5'- GGG GTA CCC GGC CAA GCT GAT CTT CCC TTA CCC A -3', +46 (reverse): 5'- GAT CGC GGC GTT GGC -3'.

The expression vector of TonEBP (Ko *et al*., 2000) and the dominant negative TonEBP (Trama *et al*., 2002) were cloned into pFLAG-CMV2 (Sigma Aldrich).

2.3. Luciferase Assay

Transient transfection into cells was performed with the calcium phosphate method by using CellPhect Transfection Kit (Amersham Bioscience, Piscataway, NJ, USA). The Renilla luciferase plasmids and pRL-thymidine kinase promoter (pRL-TK) were

co-transfected as a control. The cells were then cultured in isotonic or hypertonic medium for 24 h and harvested thereafter. The firefly and Renilla luciferase assays were performed with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

2.4. Statistical Analysis

Statistical significance was determined by Student's t-test. Each value was expressed as the mean ± S.D. Differences were considered statistically significant when the calculated P value was less than 0.05.

3. RESULTS AND DISCUSSION

3.1. Identification of TonE on Promoter Region of *TauT* Gene

It has been reported that consensus motif of TonEBP binding site is TGGAAANNYNY. We searched this motif on the *TauT* gene and then identified the consensus sequence of TonE at -110bp to -100bp from the transcriptional start site (Fig. 1A). Whereas it is complimentary from above sequence, the TonE element of urea transporter A (UT-A) gene is also so (Nakayama *et al*., 2000). As a result from sequence homology search, significant similarities were found with the upstream of mouse and human *TauT* genes (NT_039353 and NT_005927) and the TonE consensus sequence was completely conserved. Thus, the sequence may be necessary for regulation of TauT expression.

3.2. TonE Sequence Is Necessary for Osmoregulation of TauT Promoter Activity

Next, we studied the function of this *cis*-element *in vitro*. Others have produced hypertonicity by using either permeable agents, especially sodium chloride, or inpermeable agents, e.g. sucrose and mannitol. In this study, to confirm that the experimental results did not depend on permeability of the agents, we generated two kinds of hypertonic conditions by using sodium chloride (NaCl +50) and sucrose (Suc 100). When HepG2 cells were exposed to hypertonic media for 24 h, TauT expression increased at the mRNA and protein level compared with the cells cultured in isotonic medium (Iso).

To test the promoter activity of 5'-flanking region of the *TauT* gene, luciferase assay was performed in HepG2 cells (Fig. 1B, C). In the cells transfected with pTauT/-269-Luc or pTauT/-124-Luc, luciferase transcription was activated after exposed to hypertonic media. On the other hand, when the cells were transfected with pTauT/-99-Luc, which lacks TonE site, hypertonic stress did not influence luciferase expression. Furthermore, mutation of TonE sequence abolished the induction of promoter activation in response to hypertonicity, as observed in cells transfected with pTauT/-124mut-Luc. Thus, TonE consensus sequence existing from -110 bp to -100 bp of the *TauT* gene was essential to osmoregulation of TauT expression. Additionally, luciferase expression in the cells transfected with either pTauT/-99-Luc or pTauT/-124mut-Luc was markedly lower even in isotonic conditions than that in the cells transfected with pTauT/-124-Luc. These results indicate that TonE site is also required for the basal activity of TauT promoter.

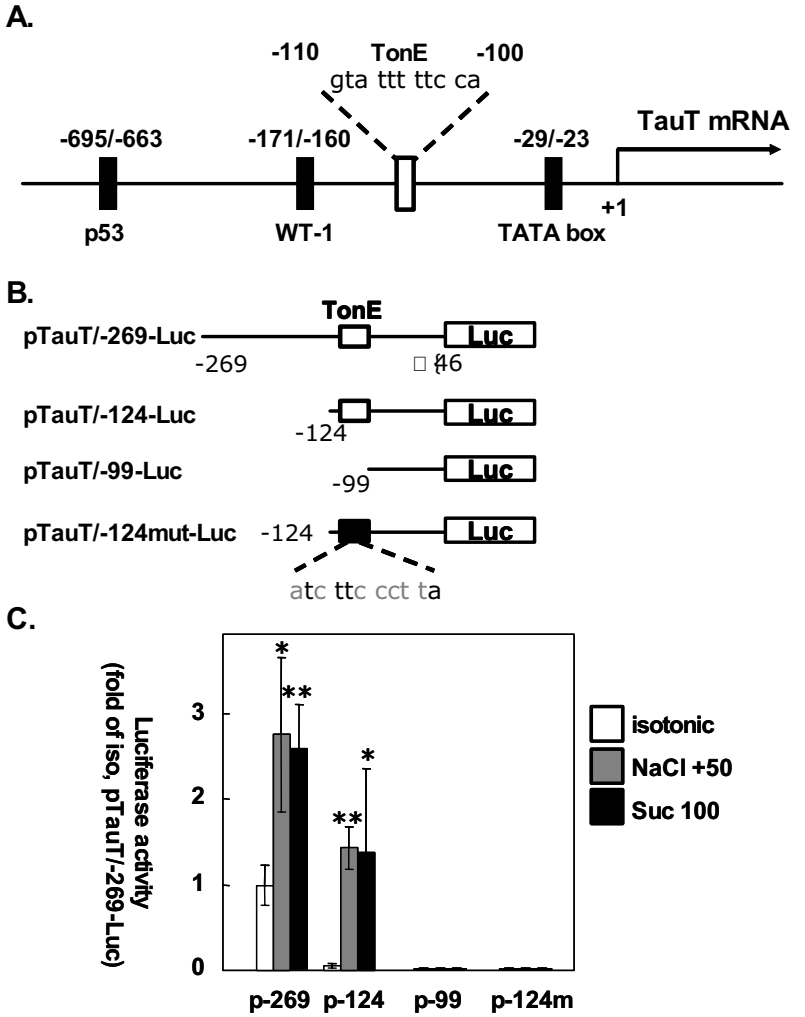

Figure 1. The role of TonE sequence in activity of TauT promoter. (A) 5'-flanking promoter region of rat *TauT* gene (AF151716) (Han *et al.,* 2000). TonE-consensus sequence is present at -110bp to -100bp upstream from transcription start site. The transcription start site is indicated by +1. The binding site for p53 and WT-1 in TauT promoter has been previously reported (Han *et al*., 2002; Han and Chesney, 2003). (B) The luciferase (Luc) constructs containing the different length of the 5'-flanking region of *TauT* gene were generated. (C) Promoter activity in transfected cells in isotonic or hypertonic (+50, Suc100) medium. Promoter activity was normalized with luciferase activity of pRL-tk. Data are means ± S.D., n=4. * P<0.05; **; P<0.01 *vs*. isotonic conditions.

3.3. Effect of TonEBP and Dominant-Negative TonEBP

In order to examine the role of TonEBP in TauT promoter activity, we tested the effect of transfection with expression vector encoding wild type TonEBP (Fig. 2A).

Coexpression of wild type TonEBP increased luciferase expression of pTauT/-124-Luc compared with that of empty vector when the cells were cultured in isotonic medium. In contrast, mutation of the TonE sequence obliterated the induction of promoter activity by coexpression of TonEBP.

On the other hand, to elucidate the role of TonEBP in osmoregulation of TauT promoter, we tested the effect of coexpression of dominant negative (DN-) TonEBP in presence of pTauT/-124-Luc (Fig. 2B). DN-TonEBP consists of DNA-binding domain of TonEBP (214-562 a.a.), which was shown to disturb the endogenous TonEBP transcriptional activity (Trama *et al*., 2002). Coexpression of DN-TonEBP in HepG2 cells decreased promoter activity in both isotonic and hypertonic conditions.

These results indicate that the TonE/TonEBP pathway plays a role in the regulation of TauT expression and its response to hypertonic condition. The upregulation of TauT expression under hypertonic environment has been reported in cells isolated various tissue, such as kidney (Uchida *et al*., 1993; Bitoun *et al*., 2001), liver (Warskulat *et al*., 1997), intestine (Satsu *et al*., 1999), brain (Bitoun and Tappaz, 2000a,b), and so on. Since TonEBP is ubiquitously expressed, the TonEBP-mediated regulation of TauT expression may be observed in these tissues.

3.4. The Biological Role of TauT in Hypertonic Environment in Liver

The liver is exposed to hypertonic stress *in vivo* under the physiological or pathological conditions, such as nutrition uptake, hydration, and hypernatremia (Haussinger *et al*., 1997). It has been reported that hypertonicity increased susceptibility to several stresses, including heat shock, ischemia, insulin, and CD95 ligand in liver or hepatocytes, which was prevented by taurine treatment (Wettstein and Haussinger, 1997; Kurz *et al*., 1998; Reinehr *et al*., 2002).

We analyzed that the effect of taurine against hypertonicity-induced cell injury in HepG2 cells. While survival cells were decreased to 0.68 ± 0.15-fold after culture in hypertonic medium (Suc 100) for 48 h, it was recovered by taurine treatment at 20 μM, as measured by MTS assay. Collectively, these findings supported that the cellular accumulation of taurine via TauT takes a significant part in the protective function of TonEBP against hypertonicity-induced cell injury.

3.5. Prospect: The Role of TonEBP/TauT Pathway in Response to Stresses in the Heart

Taurine content in heart has been reported to be changed by pathological conditions. For instance, its level markedly increases in the heart in congestive heart failure in humans and rabbits (Huxtable and Bressler, 1974; Takihara *et al*., 1986). On the other hand, large amounts of taurine are lost from the heart during ischemia and hypoxia (Schaffer *et al*., 2002). Although it was thought that these alterations are mediated by uptake through TauT from plasma and/or leakage from cardiac cells rather than synthesis from cysteine, the signal pathways involved with the activation or inactivation of these systems in the heart remain unclear. More recently, we found that treatment with anti-tumor agent doxorubicin (Dox) down-regulates TauT expression in cultured cardiomyocytes. In the luciferase assay, while luciferase expression was decreased by Dox treatment in cardiomyocytes transfected with pTauT/-124-Luc, either deletion or mutation blunted the reduction of luciferase activity (unpublished data). These findings suggest that TonEBP may be responsible for TauT expression and TauT expression may be down-regulated through inactivation of TonEBP by Dox in cardiomyocytes.

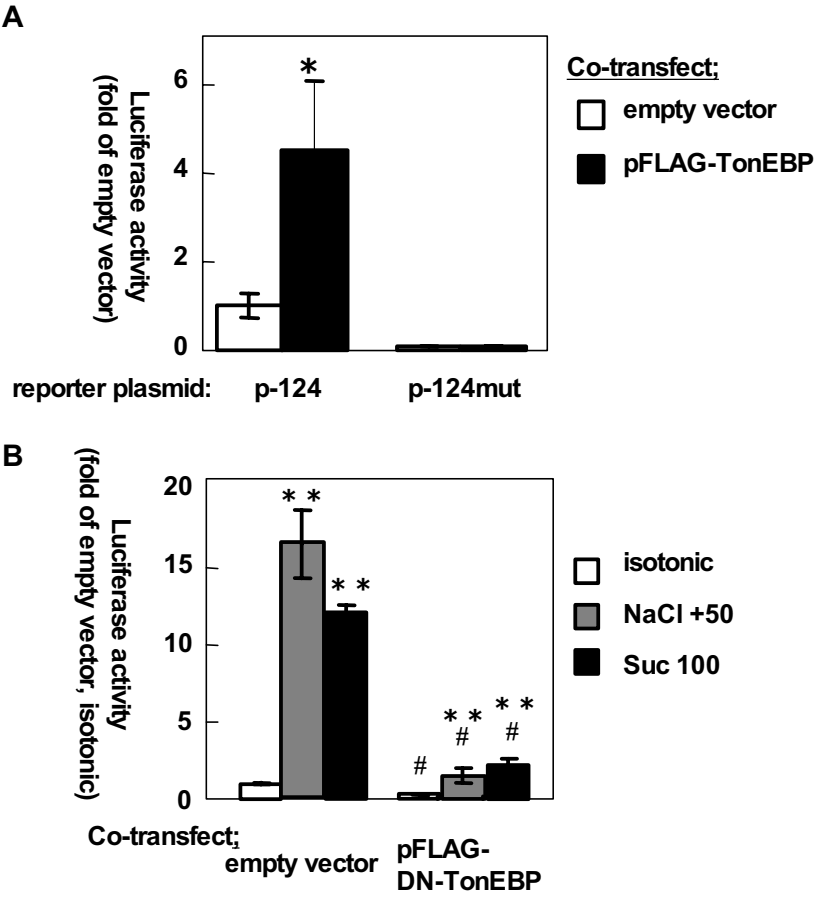

Figure 2. The effect of TonEBP or DN-TonEBP on TauT promoter activity. (A) pFLAG-TonEBP was co-transfected with pTauT/-124-Luc or pTauT/-124mut into HepG2 cells. Promoter activity was normalized with luciferase activity of pRL-tk. Data are means ± S.D., n=4. *P<0.05; **P<0.01 *vs*. control vector-transfected cells. (B) pFLAG-DN-TonEBP was co-transfected with pTauT/-124-Luc into cells, and then the cells were cultured under isotonic or hypertonic condition. Promoter activity was normalized with luciferase activity of pRL-tk. Data are means ± S.D., n=6. **P<0.01 *vs*. isotonic; #P<0.05 *vs.* control vector-transfected cells.

TonEBP is expressed in ubiquitous tissues, including the tissues, which are not exposed to high osmolality (Ho, 2003). It has been reported that the transfection with expression vector encoding DN-TonEBP in T-cells impaired the cell proliferation and increased the cell death even under isotonic condition as well as hypertonic condition (Trama *et al.*, 2002). Interestingly, the mice lacking of TonEBP die in the fetal stage (Lopez-Rodriguez *et al.*, 2004), suggesting that TonEBP is crucial for development and/or survival signals, as well as osmoregulation in many tissues. Collectively, we hypothesized that TonEBP is necessary for survival in cardiomyocytes and that the suppression of TonEBP transcriptional activity by Dox may result in cardiac toxicity. Since the function of TonEBP in heart or cardiomyocytes has not been explored, further studies will be required to reveal the essential role and the regulatory mechanism of TonEBP/TauT pathway in cardiomyocytes.

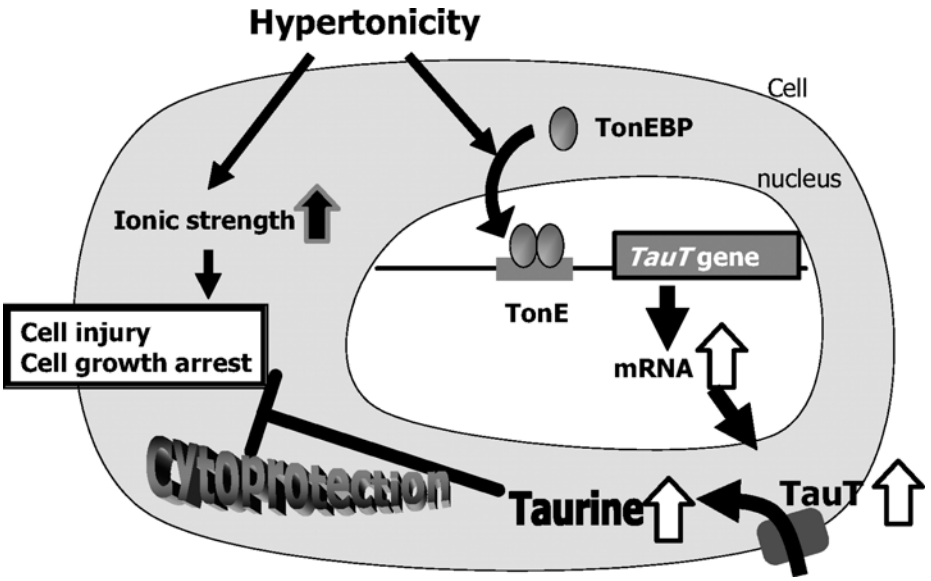

Figure 3. Scheme of pathway of TonEBP/TauT activation in response to hypertonicity. Hypertonicity rapidly induces TonEBP phosphorylation, induces its translocation into the nucleus and then activates the transcription of its target genes, including TauT.

4. CONCLUSION

In summary, this study shows that TonEBP regulates TauT expression in response to hypertonicity, and that the TonEBP/TauT pathway plays an important role in cytoprotection from hypertonic stress. The activation of TonEBP/TauT pathway could contribute to the protective signal pathway against hypertonicity-related tissue damage.

5. ACKNOWLEDGMENTS

We thank Ms. Y. Murao for her secretary work. This study was granted by Taisho Co. Ltd.

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MULTIPLE PLA_2 ISOFORMS REGULATE TAURINE RELEASE IN NIH3T3 MOUSE FIBROBLASTS

Ian Henry Lambert and Stine Falsig Pedersen[1]

1. INTRODUCTION

Most mammalian cells respond to osmotic cell swelling by activation of intracellular signaling pathways which evoke net loss of inorganic ions, compatible organic osmolytes plus cell water and subsequently restoration of the original cell volume. Here we evaluate the hypothesis that activation of a phospholipase A_2 is an initial event in the swelling-induced release of the organic osmolyte taurine from NIH3T3 mouse fibroblasts.

Taurine plays quantitatively an important role as a compatible organic osmolyte in cell volume control in mammalian cells. Within recent years it has become evident that taurine also interferes with membrane structure/function, ion channel activity, cellular Ca^{2+}-homeostasis, and cellular oxidative status, as well as with cell faith, i.e., with programmed cell death (apoptosis) (Lang *et al.*, 2003; Schuller-Levis and Park, 2003; Lambert, 2004b). The intracellular taurine concentration in NIH3T3 cells is estimated at 10 mM (Moran *et al.*, 1997) and represents a balance between active taurine uptake via the high affinity, Na^+,Cl^--dependent taurine transporter TauT and release via a volume-sensitive taurine leak pathway (Lambert, 2003a,b; Voss *et al.*, 2004). TauT expression and taurine uptake via TauT in NIH3T3 cells are down-regulated following long-term exposure to high extracellular taurine concentrations and up-regulated following deprivation of growth factors (serum starvation) and long-term exposure to TNFα (Voss *et al.*, 2004; Christensen *et al.*, 2005). Several signaling events involved in the activation of the volume-sensitive efflux pathway in mammalian cells have been revealed within recent years, and it has turned out that activation of phospholipase A_2 (PLA_2) and oxidation of arachidonic acid via the 5-lipoxygenase (5-LO) system are permissive elements for volume-sensitive taurine loss in NIH3T3 cells as well as in e.g. Ehrlich ascites tumour cells, HeLa cells, C2C12 myotubes, and pig muscle (Lambert and Hoffmann, 1993; Lambert, 1994, 1998, 2003b; Lambert and Sepulveda, 2000; Lambert *et al.*, 2001; Ørtenblad *et al.*, 2003). In contrast, lysophosphatidylcholine (LPC), which is

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Taurine 6
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a putative product of PLA_2 activity, and which induces isotonic taurine loss under isotonic conditions from a variety of cells, e.g., Ehrlich cells, NIH3T3 cells, HeLa cells, C2C12 myotubes and pig muscle cells (Lambert and Sepulveda, 2000; Lambert and Falktoft, 2000, 2001; Lambert *et al.*, 2001; Ørtenblad *et al.*, 2003), does not appear to act directly as a second messenger in the swelling-induced taurine loss from mammalian cells (Lambert, 2004b). We have previously demonstrated that swelling-activated taurine release in NIH3T3 cells is regulated by small GTP binding proteins of the Rho family, as well as by tyrosine phosphorylation events (Lambert, 2003a; Pedersen *et al.*, 2002). More recently it has been demonstrated that release of taurine from NIH3T3 is not only increased by osmotic swelling but also decreased by osmotic cell shrinkage which favors preservation of cellular osmolyte content and thereby cell volume after cell shrinkage (Pedersen *et al.*, 2005).

The PLA_2 isoform(s) involved in volume-sensitive taurine release have been investigated using a pharmacological approach. The membrane permeable lipid AACOCF3, which inhibits the cytosolic, Ca^{2+}-dependent, high molecular weight (85-110 kDa) PLA_2 ($cPLA_2$), reduces the swelling-induced taurine efflux from rat cerebral cortex (Estevez *et al.*, 1999) and from human neuroblastoma cells (Basavappa *et al.*, 1998), but has no effect on the volume-sensitive taurine efflux from NIH3T3 cells (Lambert, 2003a). RO-4639, originally designed to block pancreatic, secretory PLA_2 ($sPLA_2$) (Henderson *et al.*, 1989), reduces the swelling-induced taurine loss from Ehrlich cells as well as from HeLa cells (Lambert and Sepulveda, 2000; Lambert, 2004b). In NIH3T3 cells, the swelling-induced taurine efflux is blocked by bromoenol lactone (BEL) and potentiated by the Ca^{2+}/calmodulin antagonist W7, which has been taken to indicate that an isoform of the Ca^{2+}-independent, high molecular weight (85-90 kDa) PLA_2 ($iPLA_2$) is involved in activation of volume-sensitive taurine release in these cells (Lambert, 2003a). Thus, it appears that multiple PLA_2 isoforms are involved in swelling-induced taurine release, and that the particular isoform(s) activated depends on the cell type in question.

The mechanism(s) underlying activation of PLA_2s by osmotic stress remain incompletely elucidated, although it has been proposed that PLA_2 activation is an initial, up-stream event in the swelling-induced taurine loss from e.g. NIH3T3 cells (Pedersen *et al.*, 2002; Lambert, 2003a). Direct activation of snake venom $sPLA_2$ by osmotic swelling has been demonstrated in artifical lipid vesicles (Lehtonen and Kinnunen, 1995), but in contrast to the osmotic swelling of artificial lipid vesicles, osmotic swelling of cells may not be associated with membrane stretch, but rather with loss of membrane invaginations. Full catalytic activity of $cPLA_2$ requires Ca^{2+}-dependent translocation from the cytosol to the perinuclear membrane as well as direct phosphorylation by the mitogen activated protein kinases (MAPKs) ERK1/2 or p38 kinase (Kudo and Murakami, 2002). However, in Ehrlich ascites tumor cells, where the $cPLA_{2\alpha}$ isoform translocates to the nuclear envelope within the first minute following hypotonic exposure, there is no detectable swelling-induced change in the intracellular free Ca^{2+} concentration, $[Ca^{2+}]_i$ (Jørgensen *et al.*, 1997) and the swelling-induced translocation of $cPLA_2$ is unaffected by inhibitors of ERK1/2 and p38 kinase (Pedersen *et al.*, 2000). It is emphasized that even though changes in $[Ca^{2+}]_i$ are not involved in the initiation of the volume-sensitive, intracellular signalling cascade, an increase in $[Ca^{2+}]_i$ can still accelerate the volume regulatory response in Ehrlich cells (Jørgensen *et al.*, 1997) and taurine efflux from HeLa cells (Falktoft and Lambert, 2004). In the latter case it has recently been demonstrated

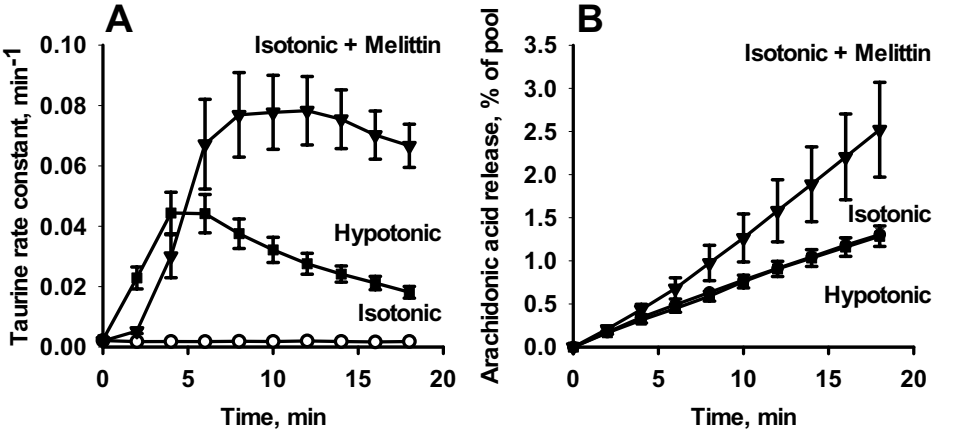

Figure 1. Swelling- and melittin-induced taurine efflux and arachidonic acid release from NIH3T3 fibroblasts. NIH3T3 fibroblasts were maintained in Dulbecco modified Eagles medium (10% heat-inactivated fetal calf serum, 100 U/ml penicillin/streptomycin, 37° C, 5% CO_2, 100% humidity incubator). The method for estimation of taurine efflux (Panel A) and arachidonic acid release (Panel B) has been described previously (Lambert, 2003a; Pedersen *et al.*, 2005). Briefly, cells were grown to 80% confluence in 6-well polyethylene dishes (9.6 cm^2 per well) and loaded with [^{14}C]taurine (80 µCi/l, 2 h) or [^{3}H]arachidonic acid (150 µCi/l, 24 h). At the end of the loading period the cells were washed five times with isotonic NaCl medium (300 mOsm: 143 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 1 mM $CaCl_2$, 0.1 mM $MgSO_4$, 10 mM HEPES, and 5 glucose, pH 7.4). Efflux and release experiments were initiated by aspiration of the medium followed by addition of one ml of experimental solution. In arachidonic acid release experiments 0.5% BSA was added to the medium in order to improve [^{3}H]arachidonic acid trapping. The cells were left for two minutes where after the medium was transferred to scintillation vial and immediately substituted by 1 ml fresh medium. This procedure was repeated for 20-24 min with a shift after 6 min (≈ time zero) to either hypotonic NaCl medium (200 mOsm: prepared as the isotonic NaCl medium but with reduced NaCl content) or isotonic NaCl medium supplemented with melittin (1.0 µg/ml). The cells were lysed at the end of the experiment with NaOH (0.5 mM), and the total tracer activity in the cell system was estimated as the sum of ^{14}C or ^{3}H activity (β-scintillation counting, Ultima Gold™ in the efflux samples, the NaOH lysate plus two final wash outs with ddH_2O. Release of taurine from NIH3T3 cells follows a mono-exponential function and the natural logarithm to the fraction of [^{14}C]taurine activity remaining in the fibroblast at the sampling time points was plotted versus time (not shown). The rate constant for the taurine efflux (min^{-1}) at each time point was estimated as the negative slope between the time point and the proceeding time point. The evolution of the rate constant for taurine release is shown as a time trace (Panel A). Arachidonic acid release, representing the contribution of several types of lipases, is shown as the total fraction released (%) plotted as a function of time (Panel B). Values are given as mean values ± SEM from 25 (isotonic, open circles), 26 (isotonic plus melittin, closed circles), 12 (hypotonic, closed squares), experiments (Panel A), and from 7 (isotonic, open circles), 7 (isotonic plus melittin, closed circles), and 7 (hypotonic, closed squares (Panel B). Data are modified from (Pedersen *et al.*, 2005).

that the Ca^{2+}-mediated effect involves a combination of calmodulin- and novel PKC-mediated potentiation of the volume-sensitive taurine efflux pathway and activation of Ca^{2+} sensitive K^+ and Cl^- channels (Falktoft and Lambert, 2004). Activation of $iPLA_2$ isoforms is reported to involve reactive oxygen species (ROS) (Martinez and Moreno, 2001; Kudo and Murakami, 2002;). However, even though ROS are generated in NIH3T3 cells following hypotonic exposure, this production appears to involve the activation of a NAD(P)H oxidase at a step down-stream to the $iPLA_2$ activation (Lambert, 2003a). Furthermore, it has been demonstrated that ROS play a role in the

modulation of the open probability of the volume-sensitive taurine release pathway in NIH3T3 cells, presumably via inactivation of protein tyrosine phosphatases and a subsequent shift in protein tyrosine phosphorylation and activity of c-Src (Lambert, 2004a).

In order to test whether a direct activation of PLA_2 could elicit taurine loss under isotonic conditions via the volume-sensitive signaling cascade we used the cationic, amphiphilic bee venom melittin. Melittin is a 26 amino acid peptide with 4 positive residues at the COOH terminus and melittin exists in its membrane bound form in at least two different functional configurations, depending on the lipid composition and the lipid to peptide ratio (Cajal and Jain, 1997). Melittin has no effect on the kinetic properties of PLA_2 but is instead reported to promote substrate replenishment by direct exchange of the product of the PLA_2 -mediated hydrolysis (Cajal and Jain, 1997). Addition of melittin to NIH3T3 cells under isotonic conditions is previously demonstrated to induce a taurine release which is blocked by inhibitors of $iPLA_2$, $cPLA_2$, 5-LO, and by a low concentration of the anion channel blocker DIDS (Lambert, 2003a). Thus, the swelling- and the melittin-induced taurine efflux share intracellular signaling elements as well as a common efflux pathway (Lambert, 2003a). The effect of melittin on taurine efflux is confirmed in Fig. 1A, where it is seen that addition of a low dose of melittin (1 μg/ml ≈ 350 nM) results in release of taurine which is, however, somewhat delayed in onset compared to the swelling-induced taurine release. From Fig. 1B it is seen that addition of melittin also results in an increase in arachidonic release, whereas hypotonic exposure does not induce any detectable increase in arachidonic acid release. It is noted that the potentiating effect of melittin on arachidonic acid release and taurine efflux is recently demonstrated to be substantially increased in osmotically swollen cells and almost abolished in osmotic shrunken cells (Pedersen *et al.*, 2005). As melittin increases the substrate availability, we have suggested that the ability of melittin to increase arachidonic acid release requires that a PLA_2 is already active (Pedersen *et al.*, 2005). It should also be noted that the lack of a detectable increase in arachidonic acid release likely reflects the subcellular localization of the PLA_2 isoforms involved, and/or a rapid metabolization of the released arachidonic acid e.g. via the 5-LO pathway (for a discussion, see Pedersen *et al*., 2005).

Melittin is reported to induce cell swelling (Dempsey, 1990) and in order to test whether the melittin-induced arachidonic acid release and taurine efflux were secondary to a melittin-induced cell swelling, we estimated the effect of melittin on cell volume, using gently trypsinised cells and electronic cell sizing. From Fig 2A it is seen that NIH3T3 fibroblasts in the presence of extracellular Na^+ swell within the first minute following addition of melittin, where after they shrink to values significantly below the original cell volume. Similar data for NIH3T3 cells have been observed using light scatter technique, and the secondary shrinkage was proposed to reflect loss of both taurine and KCl (Pedersen *et al.*, 2005). It is noted that the delayed cell shrinkage seen in the melittin-treated NIH3T3 cells (Fig. 2A) is in congruence with the delayed onset of taurine release (Figs 1A). The initial cell swelling is impaired when extracellular Na^+ is substituted by N-methyl-D-glucamine$^+$ (NMDGCl) (Fig. 2A), which is taken to indicate that the initial cell swelling is Na^+-dependent but not a prerequisite for the melittin-induced PLA_2 activity and taurine release. From Fig. 2A it is also seen that the initial

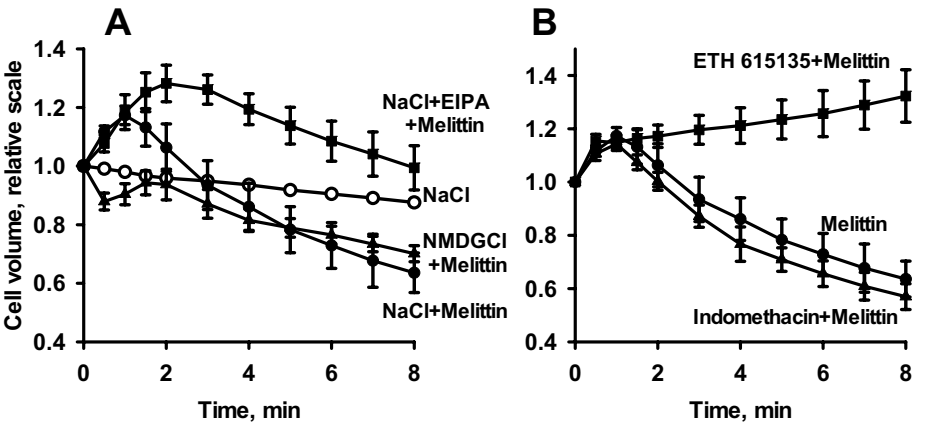

Figure 2. Melittin-induced changes in cell volume in NIH3T3 fibroblasts. Cells, grown to about 80-90% confluence in 150 cm^2 culture flasks, were detached by trypsination for 2 min at 37ºC, transferred to a sorwal glas, centrifuged gently (30 s, 700 x *g*), and resuspended in isotonic NaCl solution. Two ml of the resuspended cells were diluted 25 times in either isotonic NaCl medium (circles) or isotonic Na^+-free, N-methyl-D-glucamine-Cl medium (NMDGCl substituted for NaCl in equimolar amounts, triangles) and the cell volume estimated by electronic cell sizing in a Coulter counter. All media were filtered (0.45 μm filters). Absolute cell volumes were obtained from the median of the distribution curves after calibration with latex beads (14.1 μm diameter, Coulter Electronics). Melittin was added at time zero at a final concentration of 1 μg/ml. EIPA (5 μM), indomethacin (5 μM) and ETH 615135 (30 μM) were added in order to inhibit Na^+/H^+ exchange, cyclooxygenase and lipoxygenase activity, respectively. The cell volume is in all cases given relative to the initial cell volume, estimated within one minute before addition of melittin. Data are given as mean values ± SEM of 3 (NaCl, open circles), 6 (NaCl plus melittin, closed circles), 5 (NMDGCl plus melittin, closed triangles), 5 (NaCl with EIPA plus melittin, closed squares), 3 (NaCl with ETH 615135 plus melittin, closed squares) and 3 (NaCl with indomethacin plus melittin, closed triangles).

melittin-induced cell swelling does not involve activation of the Na^+/H^+ exchanger NHE1, since the magnitude of the swelling is increased by the NHE1 inhibitor EIPA (Fig. 2A). However, EIPA delays the unset of the subsequent shrinkage phase, indicating that the arachidonic acid release and/or the downstream intracellular signalling and osmolyte release require a functional NHE1. Whether the dependence on NHE1 reflects the alkaline pH optimum of PLA_2 (Sweatt *et al.*, 1986) or a pH dependence downstream of PLA_2 is unknown. NHE1 is activated by multiple stimuli including intracellular acidification or osmotic shrinkage, after which NHE1 serves to regulate pH_i or cell volume, respectively, back towards the normal value. In some cases however, activation of NHE1 by acidification or other stimuli can interfere with cell volume regulation or elicit significant cell swelling under isotonic conditions (Livne and Hoffmann, 1990; Pedersen *et al.*, 2003; Pedersen and Cala, 2004). It has been shown in other cell types that Cl^- recycling via the anion exchanger during RVD elicts a decrease in pH_i which activates NHE1 (Livne and Hoffmann, 1990). Thus, it seems likely that NHE1 helps to maintain pH_i during RVD within the optimal range for arachidonic acid release and/or taurine efflux. Consistent with the former notion, inhibition of NHE1 is previously demonstrated to attenuate arachidonic acid release from ischemic rat brain (Pilitsis *et al.*, 2001), and thrombin-mediated PLA_2 activation in platelets (Sweatt *et al.*, 1986). From Fig. 2B it is seen that the melittin-induced cell shrinkage, resulting from loss of taurine and KCl

(Pedersen *et al.*, 2005), is unaffected by the cyclooxygenase inhibitor indomethacine but completely abolished in the presence of the 5-LO inhibitor ETH 615-135. This is taken to indicate that the melittin-induced cell shrinkage and taurine efflux are strictly dependent on oxidation of arachidonic acid via the 5-LO system. A 5-LO metabolite, leukotriene D_4, has previously been demonstrated to induce taurine efflux from Ehrlich cells under isotonic conditions at concentrations too low to elicit Ca^{2+} mobilization (Jørgensen *et al.*, 1996; Lambert, 1998).

Most biological membranes contain a large fraction of anionic lipids (phosphatidyl glycerol, phosphatidyl serine, phosphatidic acid) and it is estimated that the affinity of melittin for anionic membranes is 1 to 2 orders of magnitude larger than the affinity towards neutral membranes (Lazaridis, 2005). Moreover, several mammalian $sPLA_2$s are cationic and exhibit marked preference for anionic phospholipids (Murakami and Kudo, 2003). From Figs. 3A and 3B it is seen that the melittin-induced arachidonic acid release and taurine efflux are significantly increased when extracellular Na^+ is substituted by $NMDG^+$ but almost impaired when extracellular Na^+ is substituted by K^+. As the plasma membrane hyperpolarizes and depolarizes when the cell is exposed to Na-free NMDGCl and KCl media, respectively, and melittin shifts lipid membranes to a more conductive state when a trans-negative potential is applied (Kempf *et al.*, 1982), it is plausible that the effect of the cation substitution on PLA_2 activity and taurine release reflects a shift in the interaction of the cationic melittin and/or PLA_2 with the anionic membrane. It is noted that the Cl^- conductance in e.g. Ehrlich cells exceeds the K^+ conductance following

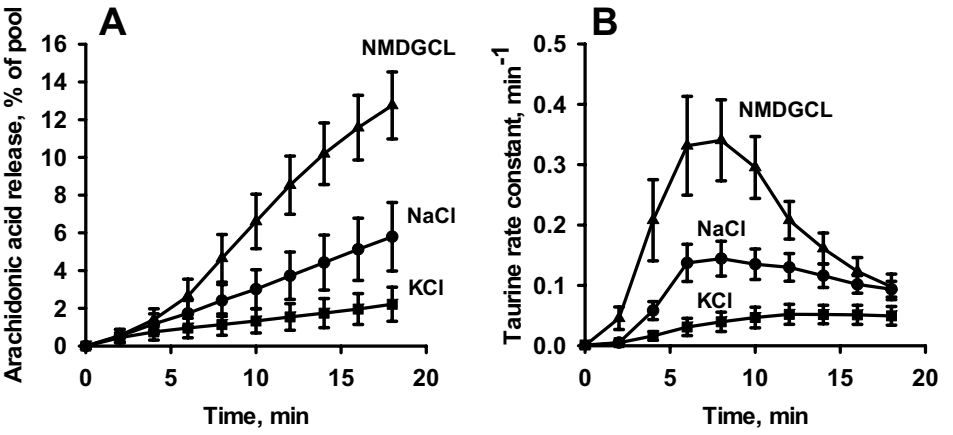

Figure 3. The effect of melittin on arachidonic acid release and taurine efflux from NIH3T3 fibroblasts is sensitive to the membrane potential. Arachidonic acid release (Panel A) and taurine efflux (Panel B) were estimated as indicated in the legend to Fig. 1 in the absence and presence of 1 μg melittin/ml in isotonic NaCl medium (circles), NMDG-Cl medium (triangles) and KCl medium (Na replaced by K in equimolar amount; squares). The time traces shown are data from melittin treated cells subtracted data from control cells. Values in Panel A and B represent 7 (NaCl), 7 (NMDGCl) and 4 (KCl) sets of experiments. Melittin increased the arachidonic acid release as well as the taurine efflux significantly (t-test, $P<0.05$, arachidonic acid release values at time 14 min tested against the control with no melittin; taurine rate constant at time 8 min, i.e., at the time of the maximal effect of melittin tested against control with no melittin).

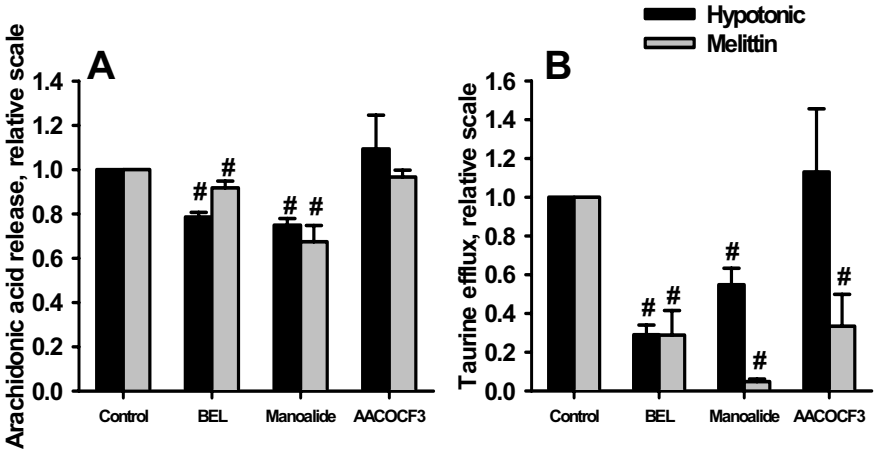

Figure 4. Role of $iPLA_2$, $cPLA_2$ and $sPLA_2$ on swelling- and melittin-induced taurine efflux and arachidonic acid release from NIH3T3 fibroblasts. Cells were prepared and arachidonic acid release and taurine efflux estimated as a function of time in either hypotonic NaCl medium (200 mOsm), isotonic NaCl medium (300 mOsm) supplemented with 0.5 μg melittin/ml. Bromoenol lactone (BEL, 30 μM), Manoalide (5 μM) and $AACOCF_3$ (40 μM) where added in order to block $iPLA_2$, $sPLA_2$, and $cPLA_2$ respectively. Arachidonic acid release and taurine efflux rate constants are given relative to control cells, i.e., cells exposed to hypotonic NaCl (200 mOsm, black bars) and isotonic NaCl supplemented with 0.5 μg melittin/ml (300 mOsm, grey bars). Arachidonic acid and taurin data are given as means ± SEM and reproduced from(Lambert, 2003a;Pedersen *et al.*, 2005). # indicates significant different from control ($P<0.05$).

osmotic cell swelling, causing the plasma membrane to depolarize (Lambert *et al.*, 1989). Such a depolarization would thus tend to limit PLA_2 binding to the membrane and could function as an off-switch for the swelling-induced signaling cascade that initiated the net loss of taurine and other osmolytes.

In order to identify the PLA_2 isoform involved in swelling- and melittin induced arachidonic acid release and taurine efflux in NIH3T3 cells, we tested the effects of the $iPLA_2$ inhibitor BEL, the $sPLA_2$ inhibitor manoalide, and the $cPLA_2$ inhibitor $AACOCF_3$. From Fig. 4 it is seen that BEL and manoalide reduce arachidonic acid release (panel A) and taurine efflux (panel B) from melittin-stimulated and hypotonically swollen NIH3T3 fibroblasts. AACOCF3, on the other hand, only reduces the melittin-induced release of taurine. These data are taken to indicate that phospholipids hydrolysis in NIH3T3 cells involves $iPLA_2$, $sPLA_2$ and $cPLA_2$ isoforms. Specifically, both $iPLA_2$ and $sPLA_2$ are involved in the volume-sensitive arachidonic acid release and the volume-sensitivity of the taurine release, whereas $sPLA_2$ activity appears to be responsible for the major fraction of melittin-induced arachidonic acid release and taurine efflux.

The present and previous studies implicate that the PLA_2 activation is an early event in cell volume sensing. The facts that *in vitro* assays indicate a complete inhibition of melittin-induced PLA_2 activity by BEL (Pedersen *et al.*, 2005), whereas *in vivo* experiments reveal only a modest effect of BEL on melittin-induced arachidonic acid release (Fig. 4A), could indicate that the localization of $iPLA_2$ in the intact fibroblast precludes activation by melittin. Using laser scanning microscopy and specific antibodies against $iPLA_2$ ($iPLA_{2\beta}$) we revealed a marked nuclear/perinuclear localization in NIH3T3

cells, as well as a punctuate localization to lamellipodia/filopodia-like regions at the plasma membrane, and vesicle-like structures (Pedersen *et al.*, 2005). The predominantly perinuclear PLA_2 localization and an intracellular release of arachidonic acid in swollen NIH3T3 cells in the absence of melittin could explain why an increased arachidonic acid release is not detectable in the extracellular medium following hypotonic exposure (Fig. 1B). The actin-based cytoskeleton, which is modulated by cell volume perturbations, has previously been demonstrated to play a role in modulation of other volume-sensitive transport pathways (Pedersen *et al.*, 1999, 2001; Jørgensen *et al.*, 2003). However, both the swelling- and melittin-induced arachidonic acid release and taurine efflux were almost unaffected by F-actin disruption using cytochalasin D (Pedersen *et al.*, 2005). Similarly, latrunculin B, which sequester actin monomers and thereby reduces actin polymerization, had no effect on either arachidonic acid release or taurine efflux (Pedersen *et al.*, 2005). Thus, taken together our findings are most consistent with a role for the membrane composition *per se* and/or a shift in the lipid packing as a volume-sensing mechanism *in vivo,* although the precise mechanism by which osmotic swelling stimulates and osmotic shrinkage inhibits. It should be noted that both swelling-activated taurine and K^+ efflux are strongly potentiated in NIH3T3 cells expressing constitutively active RhoA or Rac1 (Pedersen *et al.*, 2002), and it will be important to verify whether this reflects a role of small G proteins of the Rho family not as volume sensors *per se*, but rather in the modulation of PLA_2 activity.

2. CONCLUSION

Swelling-induced taurine efflux from NIH3T3 cells reflects a dependence on volume-sensitive arachidonic acid release. Both $iPLA_2$ and $sPLA_2$ appear to be involved in swelling-induced arachidonic acid release, likely reflecting their specific subcellular localization and dependence on lipid packing rather than volume-induced modulation of the actin-based cytoskeleton. Melittin-induced taurine efflux and cell shrinkage reflect PLA_2 activity, mainly $sPLA_2$. Both swelling- and melittin-induced taurine release is strictly dependent on 5-LO metabolites and takes place via a DIDS-sensitive leak pathway. These data are consistent with the notion that changes in PLA_2 activity are an early event in cell volume sensing in mammalian cells.

3. ACKNOWLEDGMENTS

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PROPERTIES OF VOLUME-ACTIVATED TAURINE EFFLUX FROM HUMAN BREAST CANCER CELLS

David B. Shennan, Jean Thomson, James Davidson, and Iain F. Gow[1]

1. INTRODUCTION

Cell membranes are permeable to water, which means that cell volume will be determined by the osmolality of the extracellular fluid and by the cellular content of osmotically active solutes. Although some cells can be exposed to anisosmotic conditions, most cells experience a change in their volume due to alterations in the rate of solute transport (Lang *et al.,* 1998). For example, an increase in solute uptake or an increase in solute efflux could lead respectively to cell swelling and shrinking. However, it is well established that most cells are able to regulate their volume (Hoffmann and Simonsen, 1989). Thus, if cells are placed in a hyposmotic solution they initially behave like perfect osmometers and swell, but subsequently reduce their volume. This process is known as a regulatory volume decrease (RVD) and is dependent upon a net efflux of solutes from the cells. In particular, the efflux of K^+, Cl^- and amino acids, especially taurine, underlies a RVD (Kirk, 1997). Conversely, if cells are bathed in a hypertonic solution they shrink but increase their volume towards normal. This response relies upon an increase in the cellular uptake of solutes (Hoffmann and Simonsen, 1989).

Although cells have to regulate their volumes within relatively narrow limits, it is now established that cell volume *per se* acts as a signalling system to control key metabolic processes such as protein synthesis and lipogenesis (Lang *et al.,* 1998). For example, mammary protein synthesis is stimulated by cell swelling and inhibited by cell shrinking (Millar *et al.,* 1997; Grant *et al.,* 2000). Therefore, it follows that volume-activated transport processes may have a key role to play in regulating cell growth and proliferation as a consequence of altering cell metabolism.

It is now becoming apparent that membrane transport processes, which are normally activated by an increase in cell volume, may be involved in the early stages of apoptosis. For example, volume-sensitive K^+ efflux appears to play a role in initiating programmed cell death in mouse one-cell embryos (Trimarchi *et al.,* 2002). Activation of volume-

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sensitive processes under isosmotic conditions leads to a decrease in cell volume and caspase activation. On this basis, volume-activated transport pathways may represent an important therapeutic target.

In view of the importance of volume-activated transport processes to cell survival/death we have designed experiments to investigate the effect of cell swelling on taurine efflux from human mammary cancer cell lines, namely MDA-MB-231 and MCF-7 which are respectively estrogen receptor-negative and -positive cells. In particular, we have investigated the relationship between swelling-induced taurine efflux and volume-activated anion channels together with the regulation of taurine efflux by extracellular ATP.

2. METHODS AND MATERIALS

MDA-MB-231 and MCF-7 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with L-glutamine (2 mM), heat-inactivated fetal bovine serum (10%), penicillin (50 IU/ml) and streptomycin (50 μg/ml). Both cell lines were cultured at 37°C in a gas phase of air with 5% CO_2. Cells were cultured in 75 cm^2 flasks containing 12 ml of culture medium for 4-5 days until they had reached approximately 90% confluency. The cells were then seeded in 35 mm culture wells containing 2 ml of culture medium at a density of 0.3-1.0 x 10^6 cells per well and were used 24-48 h later when they had reached 60-90% confluency.

The efflux of [^{3}H]taurine, L-[^{3}H]alanine, L-[^{3}H]leucine, α-[^{3}H]aminoisobutyric acid (AIB), ^{125}I and ^{36}Cl was measured according to the method described by Shennan *et al.* (2003). After removing the culture medium, the cells were washed three times with a buffer containing (mM) 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose, and 10 Tris-MOPS, pH 7.4. The cells were then loaded with radiolabelled solutes for 10-100 min by incubating in a buffer similar in composition to that just described except that it contained [^{3}H]taurine (1.0 μCi/ml), L-[^{3}H]alanine (1.0 μCi/ml), L-[^{3}H]leucine (1.0 μCi/ml), [^{3}H]AIB (1.0 μCi/ml), ^{125}I (2.0 μCi/ml) or ^{36}Cl (3.0 μCi/ml). After the loading period, the cells were washed five times in rapid succession with a radioactive free buffer. The efflux of the radiolabelled compounds was measured by the sequential addition and removal of 2 ml of buffer (see below for precise details) at 1 min intervals. At the end of the wash-out period, cells were lysed with 2 ml of distilled water over a period of 3 hours. The amount of radioactivity associated with the lysate was taken as a measure of the radioactivity remaining in the cells at the end of the experiment. The fractional release was calculated for each collection period as described by Shennan *et al,* (2003). This was taken as the ratio of the amount of isotope lost from the cells per minute to the amount of isotope remaining in the cells at the start of each collection period. The radioactivity left in the cells at a given time was determined by adding the amount of radioactivity left in the cells at the end of the wash-out period to the radioactivity lost by the cells in each of the 1-min collecting periods in reverse order.

The standard isosmotic solution had an osmolality of 299 mOsmol/kg water (range 295-307 mOsmol/kg water) and contained (mM) 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris-MOPS, pH 7.4. Solutions were made hyposmotic by reducing the NaCl concentration to either 75 (186 mOsmol/kg water, range 179-192) or 100 mM (235 mOsmol/kg water, range 230-240). The osmolality of all solutions was measured using a

MicroOsmometer (Vitech Scientific Ltd. UK). Purinergic agonists were dissolved directly in the isosmotic and hyposmotic solutions no more than 60 min prior to use.

Isotopes were purchased from Amersham International plc, UK. All other chemicals were obtained from Sigma, UK.

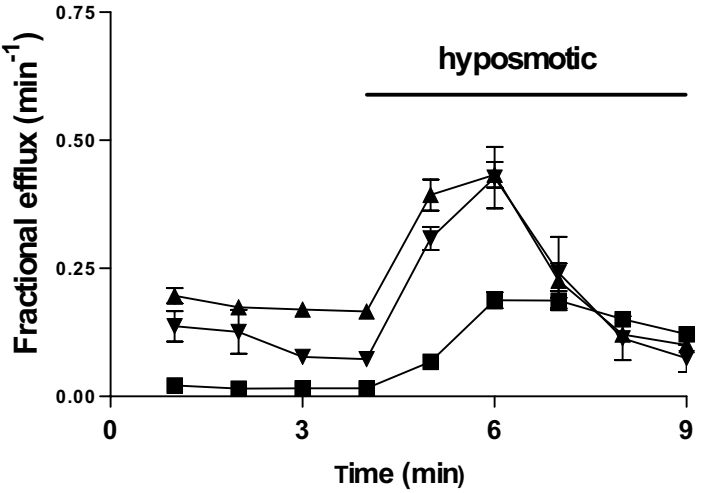

Figure 1. Effect of a hyposmotic shock on taurine (■), chloride (▲), and iodide (▼) efflux from MDA-MB-231 cells. Cells were initially incubated in a medium containing (mM) 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose, and 10 Tris-MOPS, pH 7.4. The hyposmotic solution was similar in composition except that the NaCl concentration was reduced to 75mM.

3. RESULTS

Fig. 1 shows the effect of reducing the osmolality of the incubation buffer by 38% on the fractional efflux of taurine, iodide and chloride from MDA-MB-231 cells. It is apparent that the fractional release of all three solutes was markedly increased by this manoeuvre. Whilst the profiles of hyposmotically-activated iodide and chloride efflux were similar to one another, it is notable that the profile of swelling-activated taurine efflux differed. Iodide and chloride efflux reached a peak 2 min following the osmotic shock and rapidly inactivated. On the other hand, taurine efflux reached a peak 2 min after the osmotic challenge but did not inactivate to the same extent as iodide and chloride efflux. Cell swelling also increased the fractional efflux of taurine, iodide and chloride from MCF-7 cells. Again the profiles of iodide and chloride were similar to one another but differed from that of taurine. Thus, volume-activated chloride and iodide efflux inactivated whilst that of taurine was maintained (results not shown).

Diiodosalicylate and DIDS inhibited volume-activated taurine release from MDA-MB-231 and MCF-7 cells exposed to a 38% hyposmotic challenge. Thus, diiodo-salicylate (1mM) respectively inhibited the volume-activated component of taurine efflux from MCF-7 and MDA-MB-231 cells by 96.3 ± 2.6% (±SE, n=3) and 92.1 ± 1.9% (± SE, n=5). DIDS (1mM) inhibited the fractional loss of taurine from MCF-7 cells by 94.2 ± 4.8% (± SE, n=3). However, DIDS only blocked the volume-sensitive component of

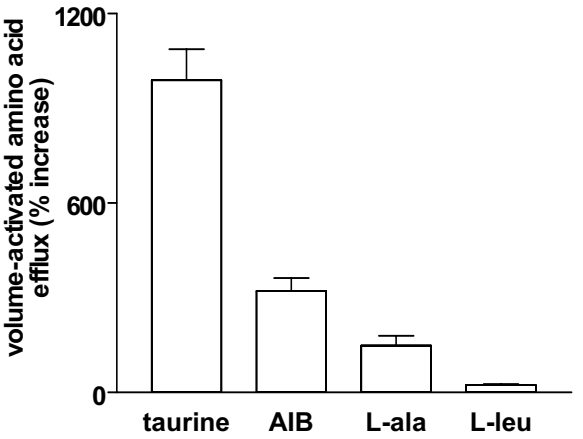

Figure 2. Effect of a hyposmotic challenge on taurine, AIB, L-alanine and L-leucine efflux from MDA-MB-231 cells. Cells were initially incubated in a medium containing (mM) 135 NaCl, 5 KCL, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris-MOPS, pH 7.4 followed by one made hyposmotic by reducing the NaCl concentration to 75 mM. Values shown for each amino acid are the differences between the fractional efflux in the isosmotic and hyposmotic medium.

taurine release by 50.2 ± 2.8% (±SE, n=3). Neither drug inhibited taurine efflux from MCF-7 and MDA-MB-231 cells under isosmotic conditions.

The effect of cell swelling on the efflux of L-leucine, L-alanine and AIB from both cell types was examined. Reducing the osmolality of the incubation medium by 38% increased the fractional loss of AIB and L-alanine, although to a lesser extent than that of taurine, from MDA-MB-231 cells (Fig. 2). A hyposmotic challenge did not significantly increase the fractional loss of L-leucine. Cell-swelling, induced by a hyposmotic shock, also increased the fractional efflux of L-alanine and AIB but not that of L-leucine from MCF-7 cells (results not shown). The profiles of volume-activated L-alanine and AIB efflux were similar to that of taurine efflux in both cell lines. Furthermore, volume-activated AIB release from MDA-MB-231 cells was inhibited by diiodosalicylate (results not shown).

Extracellular ATP increased volume-activated taurine release from MDA-MB-231 cells but not from MCF-7 cells (Fig. 3). ATP had no effect on taurine release from MDA-MB-231 and MCF-7 cells under isosmotic conditions (results not shown). Extracellular ATP also increased the volume-activated moiety of AIB efflux from MDA-MB-231 cells (results not shown). The effect of ATP on hyposmotically-induced taurine release from MDA-MB-231 cells was inhibited by short-term (5 min) Ca^{2+} removal from the incubation medium (Fig. 4). A prolonged exposure to a calcium free buffer (90 min) further decreased the volume-activated component of taurine efflux from MDA-MB-231 cells. Suramin (0.5 mM) also inhibited the swelling-induced taurine efflux from MDA-MB-231 cells: suramin respectively inhibited volume-activated taurine efflux by 75.4 ± 0.7% and 84.4 ± 2.0% (± SE, n=3) in the presence and absence of 100 μM extracellular ATP. Extracellular UTP was also effective in stimulating volume-activated taurine release from MDA-MB-231, thus, UTP (100 μM) increased volume-activated taurine release by

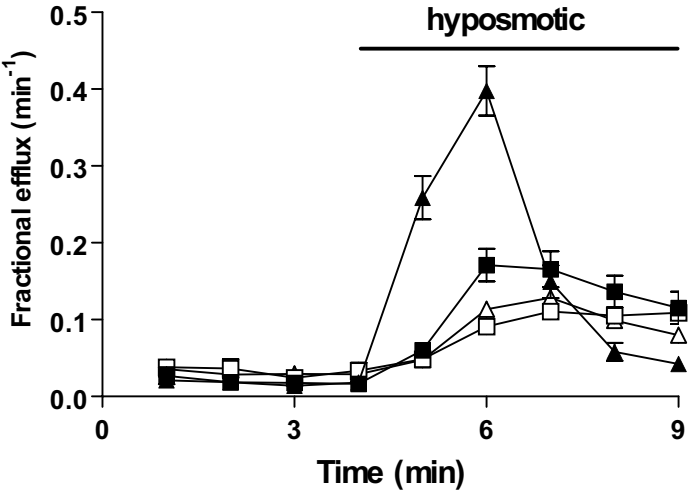

Figure 3. Effect of extracellular ATP on taurine efflux from MDA-MB-231 and MCF-7 cells. Efflux was measured from MDA-MB-231 (filled symbols) and MCF-7 cells (open symbols) in the presence (triangles) and absence (squares) of 100 μM ATP. The composition of the isosmotic and hyposmotic buffers were the same as those described in Figure 1.

443.9 ± 53.6% (± SE, n=4) when cells were exposed to a 21% hyposmotic shock. Adenosine also increased volume-activated taurine efflux from MDA-MB-231 cells but not to the same extent as ATP. Adenosine at a concentration of 100 μM increased the volume-sensitive moiety of taurine efflux from MDA-MB-231 cells by 244.8 ± 48.4% (± SE, n=4) when cells were exposed to a 21% hyposmotic challenge.

4. DISCUSSION

In this report we show that cultured human breast cancer cells, namely MDA-MB-231 and MCF-7 cells, express a volume-activated taurine efflux pathway. We wanted to determine the relationship between taurine and anion efflux as it has been suggested that volume-sensitive taurine efflux utilizes volume-activated chloride channels (see Kirk, 1997). However, this is a matter of controversy given that many studies have shown that the two pathways may be independent (e.g. see Lambert and Hoffmann, 1994; Shennan *et al.,* 1994). Volume-activated taurine efflux from MDA-MB-231 and MCF-7 cells had markedly different time courses from that of chloride and iodide: volume-activated anion efflux from both cell types inactivated at a much faster rate than taurine release. This is consistent with the notion that volume-activated taurine efflux from human breast cancer cells is not via hyposmotically-activated anion channels despite the finding that taurine efflux was inhibited by anion channel blockers such as diiodosalicylate and DIDS. It must be borne in mind that anion channel blockers are notoriously non-specific (Cabantchik and Greger, 1992).

The results suggest that the volume-activated taurine efflux pathway in MDA-MB-231 and MCF-7 cells accepts other neutral amino acids as substrates. This notion is based

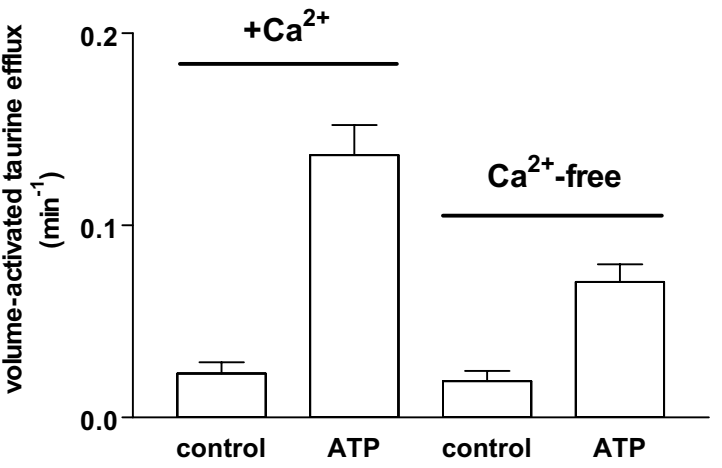

Figure 4. Effect of short-term calcium removal on the ATP-activated moeity of volume-activated taurine efflux from MDA-MB-231 cells. ATP was used at a concentration of 100 μM. The hyposmotic and isosmotic media were the same as those described in Fig. 1.

on the finding that the time course of volume-activated AIB and alanine efflux was very similar to that of taurine regarding activation/inactivation. Further studies are required to establish whether or not the swelling-activated taurine efflux pathway accepts cationic and anionic amino acids as substrates.

Extracellular ATP stimulated volume-activated taurine release from MDA-MB-231 cells in a manner which was dependent upon calcium. This is consistent with the effect of extracellular ATP on taurine release from Swiss 3T3 fibroblasts (Franco *et al.,* 2004). On the other hand Bres *et al.* (2000) have shown that extracellular ATP, albeit at high concentrations, inhibits the release of volume-activated taurine efflux from rat supraoptic glial cells. Extracellular ATP had little effect on taurine efflux from MCF-7 cells, despite the fact that MCF-7 cells express P2 receptors (Flezar and Heisler, 1993) suggesting that the volume-activated taurine efflux pathways in MCF-7 and MDA-MB-231 cells may be different entities. It appears that an influx of extracellular calcium together with calcium release from intracellular stores may contribute to the effect of ATP on taurine efflux from MDA-MB-231 cells. However, the effect of extracellular ATP on swelling-induced taurine efflux is simply not due to a rise in cytosolic calcium *per se*: ATP increases cytosolic calcium in MDA-MB-231 cells under isosmotic conditions without affecting taurine efflux (Gow *et al.,* 2005). Two findings suggest that extracellular ATP is acting via P2 receptors, possibly the P2Y2 subtype. Firstly, extracellular UTP increased swelling-activated taurine efflux and secondly, suramin blocked the effect of extracellular ATP on taurine efflux. However, we found that suramin inhibited volume-sensitive taurine release under conditions where no ATP was added to the incubation medium. This suggests that suramin may also act directly upon the transport pathway to inhibit taurine efflux. In this connection, there is evidence to suggest that ATP is released from many types of cell following a hyposmotic shock (Feranchak *et al.,* 1998; Hisadome *et al.,* 2002) although the nature of the pathway is a matter of controversy. Therefore, at

this stage, it cannot be ruled out that suramin was blocking the effect of ATP released from MDA-MB-231 cells which had been exposed to a hyposmotic shock. The effect of extracellular ATP on volume-activated taurine and K^+ efflux (Gow *et al.,* 2005) could contribute to the inhibitory effect of ATP on the proliferation of MDA-MB-231 cells previously reported by Vandewalle *et al.* (1994). We also found that adenosine stimulated taurine efflux from MDA-MB-231 cells suggesting that more than one type of receptor is involved in the regulation of swelling-activated taurine efflux. In this connection a recent report has shown that MDA-MB-231 cells express A_{2B} receptors (Panjehpour *et al.,* 2005).

It is highly probable that the volume-activated taurine efflux pathway, together with other volume-sensitive efflux pathways, contributes to breast cancer cell volume regulation and thus cell survival. Therefore, it is possible that swelling-induced efflux pathways may be potential targets to induce apoptosis. According to the hypothesis of Maeno *et al.* (2000), activation of volume-sensitive solute efflux pathways under isosmotic conditions leads to cell death.

5. ACKNOWLEDGEMENTS

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Part 2.
Metabolic Effects of Taurine

TAURINE-INDUCED CHANGES IN TRANSCRIPTION PROFILING OF METABOLISM-RELATED GENES IN HUMAN HEPATOMA CELLS HepG2

Sung-Hee Park,[1] Haemi Lee,[1] Kun Koo Park,[2] Ha Won Kim,[3] Dong Hee Lee,[3] and Taesun Park[1*]

1. INTRODUCTION

Well-known biological functions of taurine in mammalian species include stabilization of plasma membrane (Pasantes-Morales *et al.,* 1985), osmoregulation (Nieminen *et al.,* 1998), neurotransmission (Davison *et al.,* 1971), calcium modulation (Huxtable, 1987), and protein phosphorylation modulaionr (Li *et al.,* 1991). One of the best-established functions of taurine in the liver is to detoxify bile acids (Danielsson, 1963). Hypolipidemic effect of taurine has been reported in rats fed a high cholesterol diet (Gandhi *et al.,* 1992; Park *et al.,* 1998). Furthermore, taurine was effective in treating fatty liver of children with simple obesity regardless of the outcome of weight control (Obinata *et al.,* 1996). Protective effects of taurine against carbon tetrachloride or thioacetamide-induced liver damage have been shown in vivo (Balkan *et al.,* 2001; Dinçer *et al.,* 2002). Despite of accumulated knowledge on biological functions of taurine, protective mechanisms of taurine against hepatic damage or metabolic derangement such as hepatic lipid accumulation remain unclear. Taurine is provided in mammalian tissues not only by its uptake from external cells, but also by endogenous synthesis. Regulations of taurine transporter and taurine biosynthetic enzyme genes have been recently reported in the rat brain and astrocytes (Bitoun and Tappaz, 2000a,b). Although liver is the primary site of taurine synthesis, it is also the organ which shows the most variation in its concentration (4~11 μmol/g wet weight in rat liver) (Waterfield *et al.,* 1994). This variation in the level of taurine in the liver may be an important determinant of susceptibility to toxic responses.

Various techniques including Northern hybridization, Western blot and quantitative reverse transcriptase-polymerase chain reaction assay have led to the identification of

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taurine-responsive genes. Changes in the hepatic gene expression involved in biosynthesis of taurine (Schuller-Levis and Park, 2003), cholesterol metabolism (Nishimura *et al.,* 2003), cell cycle (Chen *et al.,* 2004) and anti-hypertonic response (Ito *et al.,* 2004) have been demonstrated. Although valuable, the information of gene expression assessment provided by these studies is limited when compared to what can be achieved by using a cDNA microarray technology, which allows simultaneous and coordinated expression of a large number of genes in a very short time. We have used a cDNA microarray technology that allows assessment of approximately 8,170 genes for their expression in HepG2 cells. Profiling of hepatic gene expression changes induced by taurine would provide further insights into molecular action of taurine.

2. MATERIALS AND METHODS

2.1. Cell Culture and RNA Isolation

Human hepatoma cells (HepG2) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, Gland Island, NY, USA) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Gibco-BRL, Gland Island, NY, USA), 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were incubated at 37°C in humidified atmosphere of 5% CO_2 in air. For a cDNA microarray analysis, 2.0×10^5 cells/dish were seeded into 100 mm culture dishes and cultured in serum-free DMEM for 24 h and then treated with 20 mM taurine (Sigma, St. Louis, MO, USA) for 24 h at 37°C.

Total RNA from HepG2 cells was isolated with Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration of RNA was determined from the optical density at 260 nm and the purity was ascertained from the ratio of optical density at 260 and 280 nm. The integrity of RNA was checked by running an electrophoresis on an agarose gel.

2.2. cDNA Microarray Analysis

The microarray analysis was performed according to the manufacturer's instructions (www.digital-genomics.co.kr) by using a GenePlorer TwinChip Human-8K (Digital Genomics Co., Seoul, Korea). The 8,298 genes on the array include 3,559 known genes, 4,611 unknown genes and 128 quality control probe sets. Fluorescently labeled cDNA was prepared by the reverse-transcription of total RNA in the presence of aminoallyl-dUTP followed by the coupling of Cy3 (for control cells) or Cy5 dye (for taurine-treated cells) (Amersham Pharmacia Biotech, Seoul, Korea). The cDNA microarray was hybridized at 42°C for 18 h with a mixture of fluorescently labeled cDNA from control and taurine-treated cells and then washed. cDNA chips were scanned using the ScanArray Lite (Perkin-Elmer Life Sciences, Billerica, MA, USA) and scanned images were analyzed with the GenePix 3.0 software (Axon Instruments, Union City, CA, USA) for gene expression ratios (taurine-treated *vs.* control cells). Hybridization intensity of repeated microarray experiments is expressed as means±SEM of triplicate independent experiments.

2.3. Real-Time Reverse Transcription-Polymerase Chain Reaction Analysis

We used a real-time reverse transcription-polymerase chain reaction (RT-PCR) as a complementary method to verify the results of cDNA microarray. Total RNA (1 μg) was reverse-transcribed using the Superscript™ II kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendation. Primers for the real-time PCR were designed using the Primer 3 interface available online from the Whitehead Institute for Biomedical Research. The primer sequences and GeneBank accession numbers of relevant templates are shown in Table 1. Primers were also purchased from the Bioneer Co. (Daejeon, Korea) to amplify the 530 bp cDNA fragment encoding β-actin as an internal control. Real-time PCR reactions were then carried out in a 20 μl reaction mixture (2 μl cDNA, 16 μl SYBR Green PCR Master Mix and 1 μl 0.5 μmol/l specific gene primer pair) using the LightCycler™ instrument (Roche Diagnostics Co., Indianapolis, IN, USA). The PCR reaction was carried out by 40 cycles of 95°C for 10 s, 55°C for 5 s and 70°C for 30 s. Gene expression level in each sample was analyzed according to the comparative cycle threshold method, and normalized by the β-actin expression value.

Table 1. Primer sequences and PCR conditions

Primer	Acession No (NCBI)	Primers	Sequences (5' -> 3')	PCR product (bp)	Annealing (℃)
β-Actin	—	F	GTGGGGCGCCCCAGGCACCAGGGC	530	54
		R	CTCCTTAATGTCACGCACGATTTC		
GPAT	AFO43937	F	GTGATTCTGAAAGTGGACTC	137	52
		R	GCTACTGCTACTAAGGATGG		
BCKADH	AF026548	F	CCTGAACTGTGAGCTGATT	154	52
		R	GGAGTGTCTTGTGAAGTCAT		
BCAT2	U68418	F	ACAGAGTCTACTGGACATGG	172	52
		R	CCTGTCTTTGTACAGGATTC		
PDHK	U54617	F	TAACTGTGATGTGGTAGCAG	157	52
		R	GAGGTGAGAAGGAACATACA		

GPAT, glyceronephosphate O-acyltransferase; BCKADH, branched-chain keto acid dehydrogenase E1, beta polypeptide; BCAT2, branched chain aminotransferase 2, mitochondrial; PDHK, pyruvate dehydrogenase kinase, isoenzyme 4.

3. RESULTS

3.1. Changes in Hepatic Gene Expression Profile Involved in Metabolism

Changes in the hepatic gene expression profile of HepG2 cells treated with taurine were assessed using cDNA microarray analyses. Of 8,298 genes on the GenePlorer TwinChip Human-8K microarray used in this study, 4,837 genes (59.2%) were identified as present in HepG2 cells. Of these genes, 477 genes underwent a greater than two-fold change in taurine-treated cells compared with the control cells. Among these, 128 genes were up-regulated and 349 were down-regulated more than two-fold by taurine treatment.

Among 128 up-regulated and 349 down-regulated genes, known genes were counted as 87 and 206, respectively (Fig. 1). After clustering regulated genes based on biological function, we found that taurine up-regulated mainly genes that are implicated in the processes of amino acid metabolism, fatty acid metabolism, cytoskeletal activity, ion channel, signal transduction, cell proliferation and DNA repair. On the other hand, taurine down-regulated mainly genes that are related to lipid metabolism, proteolysis, immune response, transcription, signal transduction, apoptosis and cell proliferation.

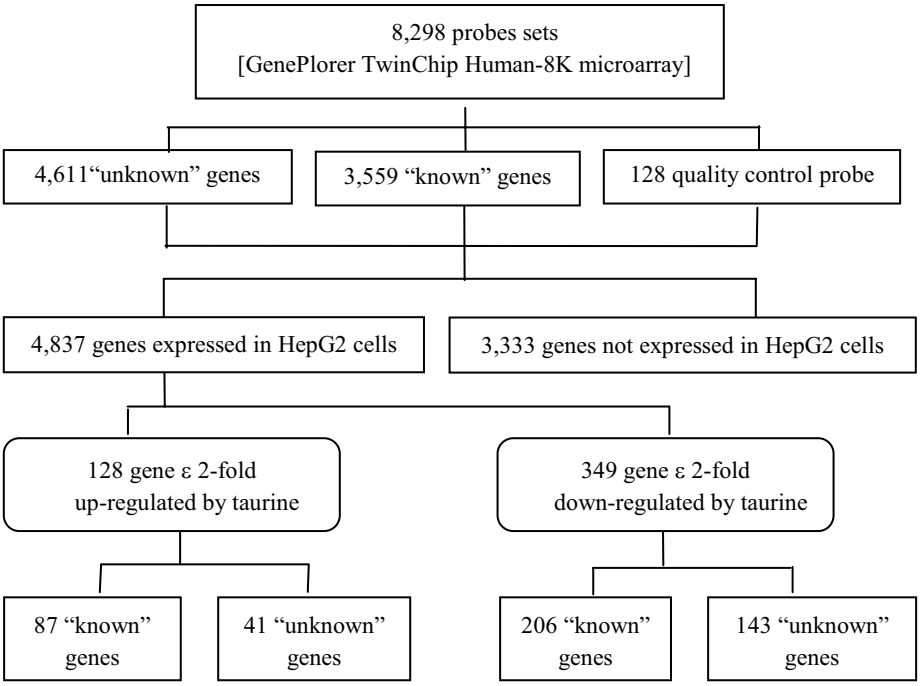

Figure 1. Diagrammatic representation of the analysis of genes tested in the current study.

Tables 2, 3 and 4 list the known taurine-responsive genes, whose major biological function is categorized into metabolism. Among taurine-responsive genes involved in carbohydrate metabolism, two genes were up-regulated more than four-fold and six genes were down-regulated more than two-fold (Table 2). For taurine responsive genes involved in lipid metabolism, glyceronephosphate O-acyltransferase and myotubularin related protein 4 genes were up-regulated 3.1- and 2.2-fold, respectively and six genes were down-regulated more than two-fold (Table 3). Among taurine responsive genes involved in protein or amino acid metabolism, four genes were up-regulated more than 1.5-fold and ten genes were down-regulated more than two-fold (Table 4). Interestingly, products of those four genes up-regulated by taurine (branched chain aminotransferase 2, branched-chain aminotransferase 1, branched-chain keto acid dehydrogenase E1, and HMG-CoA lyase) were all involved in branched-chain amino acid catabolism.

3.2. Confirmation of Microarray Results by Real Time RT-PCR

Microarray results were verified by conducting real time RT-PCR analyses using identical RNA samples. We selected genes related to metabolism (glycerophosphate O-acyltransferase, branched chain keto acid dehydrogenase E1, beta polypeptide, branched chain aminotransferase 2, mitochondrial, and pyruvate dehydrogenase kinase isoenzyme 4) with varying expression profiles for real time RT-PCR analyses. The results of real-time RT-PCR analyses of these selected genes were consistent with our cDNA microarray data; although the fold changes in the expression level differed somewhat in the two analytical methods (Fig. 2). These results support the findings obtained from our microarray experiments and also suggest that taurine regulates the transcription of genes that are related to metabolism in the liver.

Table 2. Taurine responsive genes related to carbohydrate metabolism in HepG2 cells

Accession no.	Gene description	Signal ratio (test/cont)	
		Mean	SEM
AI672108	UDP-glucose pyrophosphorylase 2	4.6	1.0
Y08136	Sphingomyelin phosphodiesterase, acid-like 3A	4.1	1.7
AI289196	Mannosidase, alpha, class 1A, member 2	0.4	0.0
U85773	Phosphomannomutase 2	0.4	0.1
AA203426	KIAA1838	0.3	0.1
U54617	Pyruvate dehydrogenase kinase isoenzyme 4	0.3	0.0
AI767809	Mannosidase, alpha, class 2A, member 1	0.2	0.1
NM_002711	Protein phosphatase 1 regulatory (inhibitor) subunit 3A (glycogen and sarcoplasmic reticulum binding subunit, skeletal muscle)	0.2	0.0

Hybridization intensity normalized to *Sacharomyces cerevisae* intergenic sequence mRNA expression of repeated microarray experiments were expressed as mean and SEM of triplicate independent experiments.

4. DISCUSSION

HepG2 cells express proteins involved in cholesterol and triglyceride metabolism (Javitt, 1990) and secrete many human plasma proteins (Knowles *et al.,* 1980). Both HepG2 cells and freshly isolated human hepatocytes have similar glucuronidation and cytochrome reductase activities, but differ in microsomal epoxide hydrolase activity (Grant *et al.,* 1988). Taurine-responsive genes identified from our cDNA microarray analyses do not obviously encompass the whole profile of genes involved in carbohydrate, lipid and protein/amino acid metabolism, since the cDNA chip used in the current study holds 8,170 genes which account for only 20% of total genes expressed in human tissues.

Table 3. Taurine responsive genes related to lipid metabolism in HepG2 cells

Accession no.	Gene description	Signal ratio(test/control)	
		Mean	SEM
AF043937	Glyceronephosphate O-acyltransferase	3.1	0.3
AB014547	Myotubularin related protein 4	2.2	0.6
NM_002660	Phospholipase C, gamma 1	0.4	0.1
AI767533	Phosphate cytidylyltransferase 1, choline, alpha isoform	0.3	0.1
AI913528	Selenoprotein I	0.3	0.1
X87176	Hydroxysteroid (17-beta) dehydrogenase 4	0.2	0.2
AA993882	Geranylgeranyl diphosphate synthase 1	0.1	0.0
J02883	Colipase, pancreatic	0.1	0.0

Hybridization intensity normalized to *Sacharomyces cerevisae* intergenic sequence mRNA expression of repeated microarray experiments were expressed as mean and SEM of triplicate independent experiments.

Table 4. Taurine responsive genes related to protein or amino acid metabolism

Accession no.	Gene description	Signal ratio(test/cont)	
		Mean	SEM
U68418	Branched-chain aminotransferase 2, mitochondrial	2.1	0.5
AI970531	Branched-chain aminotransferase 1, cytosolic	1.6	0.0
AI795940	Branched-chain keto acid dehydrogenase E1, beta polypeptide	2.2	0.1
NM_000191	HMG-CoA lyase	1.8	0.2
AB007887	KIAA0427	0.4	0.1
U15932	Dual specificity phosphatase 5	0.4	0.0
X66362	PCTAIRE protein kinase 3	0.4	0.0
AK022339	Seryl-tRNA synthetase	0.3	0.1
M98252	Procollagen-lysine 1, 2-oxoglutarate 5-dioxygenase 1	0.3	0.0
X95648	Eukaryotic translation initiation factor 2B, subunit 1 alpha, 26kDa	0.3	0.1
NM_000353	Tyrosine aminotransferase	0.2	0.0
Y18483	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 8	0.2	0.0
AI249145	N-acetylneuraminate pyruvate lyase (dihydrodipicolinate synthase)	0.1	0.0
AI884353	Mitochondrial translational release factor 1	0.1	0.1

Hybridization intensity normalized to *Sacharomyces cerevisae* intergenic sequence mRNA expression of repeated microarray experiments were expressed as mean and SEM of triplicate independent experiments.

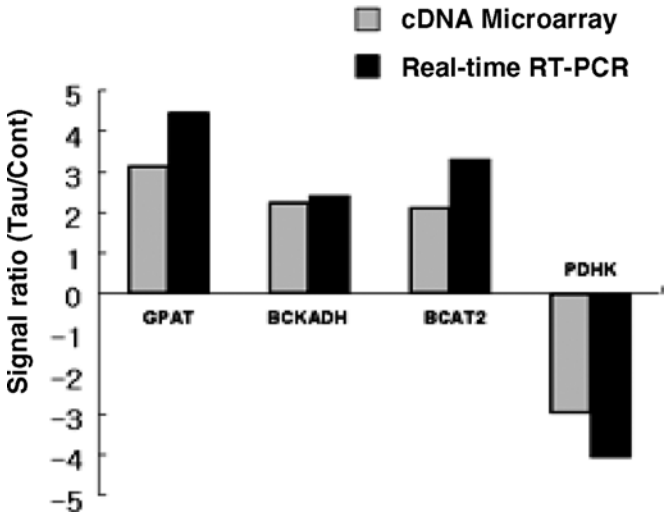

Figure 2. Confirmation of cDNA microarray results by real-time RT-PCR analyses**.** Results are expressed as the fold changes normalized to β-actin mRNA expression. GPAT, glyceronephosphate O-acyltransferase; BCKADH, branched-chain keto acid dehydrogenase E1, beta polypeptide; BCAT2, branched chain aminotransferase 2, mitochondrial; PDHK, pyruvate dehydrogenase kinase, isoenzyme 4.

Two carbohydrate metabolism-related genes up-regulated by taurine are involved in glycogen metabolism in hepatocytes. UDP-glucose pyrophosphorylase 2 catabolizes the transfer of a glucose moiety from glucose-1-phosphate to MgUTP, forming UDP-glucose and MgPPi. Since UDP-glucose is a direct precursor of glycogen in the liver and muscle tissues, taurine-induced up-regulation of UDP-glucose pyrophosphorylase 2 expression might be related to enhanced glycogen synthesis in taurine-treated hepatocytes. Protein phosphatase 1 (PP1), regulatory subunit 3A binds to muscle and liver glycogen with high affinity and enhances dephosphorylation of glycogen synthase and glycogen phosphorylase. Both glycogen synthase and glycogen phosphorylase are tightly regulated *via* phosphorylation. Glycogen synthase become active when phosphorylated, while glycogen phosphorylase become inactive when dephosphorylated. Our result of decreased PP1 expression by taurine indicates that taurine appears to activate glycogen synthase but inactivate glycogen phosphorylase. This result, taken together with the increased UDP-glucose pyrophosphorylase 2 expression by taurine, led us to hypothesize that taurine enhances glycogen synthesis in the liver.

Pyruvate dehydrogenase kinase (PDK) inactivates pyruvate dehydrogenase complex (PDC) by phosphorylating serine residues of the E1α component of the complex. Four isoenzymes of PDK have been identified in the human genome, and the expression level of pyruvate dehydrogenase kinase, isoenzyme 4 (PDK4) is a major determinant of PDK activity (Sugden *et al.,* 2000). PDC catalyzes the conversion of pyruvate to acetyl-CoA, the precursor for fatty acid synthesis and energy production *via* the TCA cycle. In the well-fed state, the PDC is relatively active and generates acetyl-CoA, while in the starved state PDC is relatively inactive, enabling the body to conserve three-carbon compounds (pyruvate, lactate, and alanine) for gluconeogenesis (Sugden *et al.,* 1989). The latter

helps to maintain euglycemia during starvation but exacerbates hyperglycemia in diabetes (Harris *et al.,* 2001). Huang *et al*. (2002) have shown that glucocorticoids and peroxisome proliferator-activated receptor α (PPARα) ligands induce PDK4 expression and insulin inhibits these effects in a rat hepatoma cell line. Regulation of PDC activity is therefore important for the control of glucose and lipid metabolism. Taurine-induced down-regulation of hepatic PDK4 gene expression observed in the current study leads to the hypothesis that taurine increases PDC activity, generating acetyl-CoA for further oxidation of three-carbon compounds via TCA cycle.

Our results of cDNA-microarray and real-time RT-PCR indicate that taurine up-regulated the expression of four enzymes involved in branched-chain amino acid (BCAAs) catabolism. Branched-chain amino acid aminotransferases (BCAT) are pyridoxal phosphate-dependent enzymes that catalyze reversible transamination of the L-branched-chain amino acids to their respective α-keto acids. Branched-chain aminotransferase 2 mitochondrial gene and branched-chain aminotransferase 1 cytosolic gene encode the mitochondrial and cytosolic forms of the enzyme branched-chain amino acid transaminase, respectively. Branched-chain keto acid dehydrogenase is involved in oxidative decarboxylation and subsequent dehydrogenation of branched-chain α-keto acid. 3-Hydroxy-3-methylglutaryl-CoA lyase (HMG-CoA lyase) is a mitochondrial matrix enzyme that catalyzes the cleavage of HMG-CoA to acetoacetic acid and acetyl-CoA; the last step of both ketogenesis and leucine catabolism. It is believed that BCAAs contribute to energy metabolism during exercise as energy sources and substrates to expand the pool of tricarboxykic acid cycle intermediates (Shimomura *et al.,* 2004). It is hypothesized that taurine improves the capacity of BCAA oxidation in the liver, which could be beneficial under the circumstances of enlarged hepatic pool of BCAAs provided as dietary supplements. Whether taurine accelerates the contribution of BCAAs as energy sources during exercise needs to be confirmed in the skeletal muscle.

Tyrosine aminotransferase is present in the liver and catalyzes the conversion of L-tyrosine into *p*-hydroxyphenylpyruvate. Procollagen-lysine 1,2-oxoglutarate 5-dioxy genase 1 (lysyl hydroxylase) catalyzes the formation of hydroxylysine in collagens and other proteins with collagen-like amino acid sequences, by the hydroxylation of lysine residues in X-lys-gly sequences. The resultant hydroxylysyl groups are the attachment sites for carbohydrates in collagen and thus critical for the stability of intermolecular cross-links. Physiological significance of the taurine-induced down-regulation of tyrosine aminotransferase or lysyl hydroxylase is not clear at the present time.

5. CONCLUSIONS

Taurine-induced changes in expression profiling of HepG2 cells were assessed using a cDNA microarray technology and confirmed by real time RT-PCR analyses. Among known genes regulated by taurine, 87 genes were up-regulated and 206 genes down-regulated more than two-fold. Among these 293 taurine-responsive genes, 30 genes were implicated in the processes of carbohydrate, lipid or protein/amino acid metabolism. Taurine-induced up-regulation of UDP-glucose pyrophosphorylase 2 and down-regulation of protein phosphatase 1 regulatory subunit 3A expressions lead to a hypothesis that taurine enhances glycogen synthesis in the liver. Taurine-induced down-regulation of

hepatic pyruvate dehydrogenase kinase isoenzyme 4 expression suggests that taurine increases pyruvate dehydrogenase complex activity, generating acetyl-CoA for further oxidation of three-carbon compounds via TCA cycle. Our results of cDNA-microarray and real-time RT-PCR also indicated that taurine up-regulated expressions of four enzymes involved in branched-chain amino acid catabolism (branched-chain amino acid aminotransferases 2; mitochondrial, branched-chain aminotransferase 1, cytosolic; branched-chain keto acid dehydrogenase; 3-hydroxy-3-methylglutaryl-CoA lyase). Our microarray results of hepatic gene expression profiling would provide further insights into molecular actions of taurine in metabolic regulations.

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THE IMPORTANT ROLE OF TAURINE IN OXIDATIVE METABOLISM

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1. ABSTRACT

Several studies have demonstrated that especially high taurine concentrations are found in tissues with high oxidative activity, whereas lower concentrations are found in tissues with primary glycolytic activity. Based on such observations, we have studied if taurine is involved in mitochondrial oxidation. Several pieces of information have demonstrated taurine localisation in the mitochondria. We have developed a general biochemical model with preliminary data demonstrating the important role of taurine as mitochondrial matrix buffer for stabilising the mitochondrial oxidation. The model can have far-reaching perspectives, e.g., explaining the often-suggested anti-oxidative role of taurine, in contrast to the fact that taurine is very difficult to chemically oxidise. By stabilising the environment in the mitochondria, taurine will prevent leakage of the reactive compounds formed in the reactive mitochondrial environment and thus indirectly act as an antioxidant. Consequently, the model represents a new concept for understanding mitochondrial dysfunction by emphasising the importance of taurine for providing sufficient pH buffering in the mitochondrial matrix.

2. INTRODUCTION

Taurine is found in all animal cells typically in millimolar concentrations, e.g., 5-50 mM (Jacobsen and Smith, 1968), whereas the concentration in plasma and extracellular fluids is much lower, typically 50-200 μM. Several different actions have been ascribed to taurine, e.g. bile acid conjugation and as an intracellular osmolyte. However, despite the ubiquitous distribution and several reviews on the action of taurine in physiology and pathophysiology (Huxtable, 1992; Hansen, 2001, 2003), the overall role of taurine is still disputed.

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3. OXIDATIVE TISSUES AND TAURINE

When considering the tissue localisation of taurine, it is evident that the highest concentrations are found in highly energy-consuming tissue, like retina, nerves, kidney, heart, and oxidative muscle tissue in general (Jacobsen and Smith, 1968). Several studies exist on taurine distribution in muscles comparing oxidative and glycolytic muscle types or fibre types (e.g. Aristoy and Toldrá, 1998; Cornet and Bousset, 1999). In the reports from such studies, the differences in distribution of taurine, carnosine, and anserine are conspicuous (see Table 1).

Table 1. Carnosine, anserine, and taurine content in glycolytic and oxidative porcine muscles

Compound	**Glycolytic muscle** *Longissimus dorsi*	**Oxidative muscle** *Masseter*
Carnosine (β-alanyl-L-histidine)	10-15 µmol/g	1-2 µmol/g
Anserine (β-alanyl-L-1-methylhistidine)	1-3 µmol/g	1-3 µmol/g
Taurine	1-3 µmol/g	15-20 µmol/g

The concentrations are adapted from Aristoy and Toldrá (1998) and Cornet and Bousset (1999).

Carnosine and anserine are recognised as an intracellular buffer for buffering lactate formed by glycolysis and is obviously found in high concentration in the glycolytic muscle and low concentration in the oxidative muscle. On the contrary, taurine is found in high concentrations in the oxidative muscle and low concentration in the glycolytic muscle. Such preferential localisation of taurine in oxidative tissue indicates a possible importance for mitochondrial function.

4. SUBCELLULAR DISTRIBUTION OF TAURINE

The concentration gradient across the cellular membrane clearly demonstrates the action of the ATP-dependent taurine transporter. However, as seen from the reviews on taurine distribution, the taurine concentrations vary significantly among the different tissue types. Although the activity of the taurine transporter might depend on the specific tissue or the cell type within the tissue, it seems more likely to explain the variation in taurine distribution by applying a two- (or multi-) compartment model. In such a model different taurine concentrations are allowed in the subcellular compartments due to up-concentration in specific organelles. The total taurine concentration in the tissue will thus depend on the organelle distribution within the cells in the tissue. Such a multi-compartmental model requires subcellular taurine transporter activity, as found in a recent study (Voss *et al*., 2004) on the recognition of taurine transporter with primary antibodies. However, no association with any specific organelle was demonstrated.

5. MITOCHONDRIAL LOCALISATION

Immunocytochemical techniques have been developed to study tissue and subcellular distribution of taurine. Such studies demonstrated that taurine was distributed in all cellular subcompartments. However, in some organelles including the mitochondria increased immunoreactivity was reported indicating taurine localisation in the mitochondria (e.g. Ottersen, 1988; Terauchi and Nagata, 1993, Lobo *et al*., 2000).

Recent studies on mitchondrial tRNA have demonstrated the existence of taurine-modified uridine residues (Suzuki, 2002). Such t-RNA modification must be expected to be processed inside the mitochondrial matrix. The taurine modifications were not found in mutant tRNA from patients with mitochondrial encephalopathies, i.e., the study indicates the importance of taurine for having normal mitochondrial function. Possibly, taurine could be directly involved in the metabolic regulation of the glucose metabolism as indicated by the results on the interaction between taurine and pyruvate dehydrogenase phosphatase (Lombardini, 1997).

6. INTRACELLULAR BUFFERS AND MITOCHONDRIA

Any discussion on mitochondrial function and mitochondrial oxidation in modern biochemical textbooks is focused on the importance of the pH gradient between the cytosol and the mitochondrial matrix. Energy is stored electrochemically in the pH gradient in order to produce ATP by the ATP synthase enzyme system. However, as a result of the pH gradient the mitochondrial matrix is mildly alkaline (see Fig. 1).

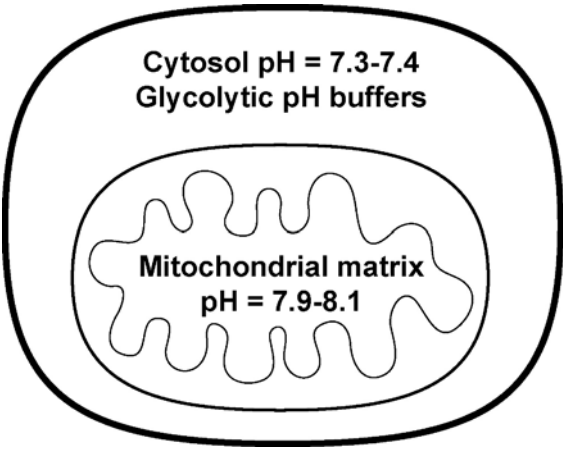

Figure 1. Schematic overview of cell indicating the pH gradient between the cytosol and the mitochondrial matrix. pH values adapted from Llopis *et al*. (1998).

The pH value of the matrix has been determined to about 8 by advanced measurements applying confocal microscopy and subcellular targeting of pH indicators based on green fluorescent protein (GFP) (e.g. Llopis *et al*., 1998).

Whereas the pH control of the cytosol by glycolytic buffers like carnosine and anserine has been described (e.g. Aristoy and Toldrá, 1998; Cornet and Bousset, 1999), no discussion on pH buffering of the alkaline pH in the mitochondrial matrix seems to exist. In Table 2 ionisation constants are presented for some compounds, which are involved in physiological pH regulation, and taurine.

Table 2. Ionisation constants for some intracellular pH buffers and taurine

Compound	pK value	Reference
Carbon dioxide/Bicarbonate (CO_2/HCO_3^-)	6.35 (25°C)	Beynon and Easterby, 1996
Dihydrogenphosphate/Hydrogenphosphate ($H_2PO_4^-/HPO_4^{2-}$)	7.21 (25°C)	Beynon and Easterby, 1996
Carnosine	6.8 (22°C)	Deutsch and Eggleton, 1938
Anserine	7.0 (22°C)	Deutsch and Eggleton, 1938
Taurine (amino group)	9.0 (25°C) 8.6 (37°C)	Hansen *et al*., 2005

It must be expected that pH buffering is required for the mitochondrial matrix due to the mildly alkaline environment inside. Existence of a low-molecular pH buffer will stabilise the mitochondrial pH gradient. Among the compounds presented in Table 2 taurine is the only candidate due its pK value. When comparing with other lists of physiological buffer, the lack of compounds with the pK values in the range 8–9 is conspicuous. In addition, the mitochondrial proteins are not expected to provide adequate buffering contribution, as the protein amino acid residues does not have pK values in this range either.

To summarise our analysis and observations, we now propose the following hypothesis on a very important cell physiological role of taurine:

Hypothesis: Taurine acts as a pH buffer in the mitochondrial matrix and thus stabilises the mitochondrial pH gradient

Preliminary experimental arguments for this hypothesis follow below. Additional thorough theoretical and experimental arguments can be found below and elsewhere (Hansen *et al*., 2005).

7. MITOCHONDRIAL MATRIX ENZYMES

In order to obtain some experimental evidence for the hypothesis proposed, the pH dependence of some enzymes localised in the mitochondrial matrix was studied. The pH dependence of isocitrate dehydrogenase from the tricarboxylic acid cycle has been determined with the results as shown in Table 3.

Table 3. pH dependence of the isocitrate dehydrogenase activity at 37°C comparing applicaton of Tris and taurine as buffer compounds

pH	Taurine	Tris
7.5	95.6	96.7
7.7	100.0	96.7
7.9	98.4	94.8
8.1	92.2	88.6
8.3	84.3	79.8
8.5	73.9	74.4
8.7	62.6	64.5
8.9	46.8	54.6
9.1	30.7	43.7
9.3	10.0	27.5
9.5	2.7	16.8

The measurement of isocitric dehydrogenase activity was performed in a final volume of 3.0 ml using a Shimadzu UV-1601 spectrophotometer. The assay was carried out as described (Bergmeyer, 1974) in a medium containing 80 mM Na_2SO_4, 0.44 mM DL-isocitric acid, 0.5 mM β-nicotinamide adenine dinucleotide phosphate, 0.2 mM manganese chloride, 0.015 U/ml isocitric dehydrogenase (from pig heart) (Sigma), and for pH buffering 40 mM of taurine or Tris. pH of the medium was adjusted to the indicated pH with H_2SO_4 or NaOH at 37°C. The activity was determined from the linear increase in absorbance at 355 nm (NADPH). Data are reported as mean of 6-8 determinations and normalised relatively to the maximum activity observed.

As seen from the data in Table 3 no significant difference is observed in enzyme activity using taurine instead of the traditional research buffer Tris. Actually, it seems that taurine inhibits enzyme activity at pH > 9 better than Tris.

Additional examples on important metabolic enzymes localised in the mitochondrial matrix are the acyl-CoA dehydrogenase enzymes (ACADs), which are primarily responsible for performing the fatty acid β-oxidation. The original mechanistic studies on these enzymes demonstrated strong pH activity dependence favouring mildly alkaline conditions at pH 8.0-8.5 (Reinsch *et al*., 1980, Schmidt *et al*., 1981) as seen in Fig. 2. In addition, to maintain a reasonably constant enzyme activity, the steep activity increase with pH immediately demonstrates a requirement for buffering the enzyme environment, i.e. buffering of the mitochondrial matrix.

Additional information on the ACADs can be found in recent studies (Ghisla and Thorpe, 2004, Hansen *et al.*, 2005). Several different ACADs were studied with different substrate specificity depending on fatty acid chain length. These studies demonstrated similar activity profiles to the profile in Fig. 2, i.e., mildly alkaline pH is required for having reasonable fatty acid β-oxidation activity. In case of insufficient pH buffering, reduced enzyme activity must be expected, and, consequently, impaired mitochondrial fatty acid oxidation will be observed.

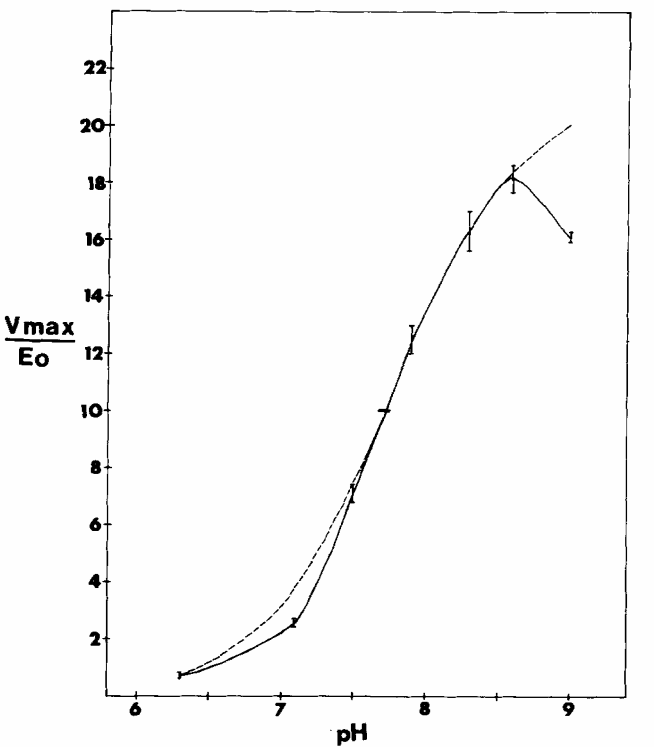

Figure 2. pH dependence of fatty acyl-CoA-dehydrogenase activity monitored through the reduction of electron transfer flavoprotein at 25°C. Phosphate buffer (pH 6.5 and 7.0) or Tris buffer (pH > 7.5) was used for the assay. The figure is reproduced from Schmidt *et al.* (1981), in which the experimental procedures are described in details.

8. ANTIOXIDATIVE ROLE OF TAURINE AND FURTHER PERSPECTIVES

Often taurine has been presented as an antioxidant despite the fact that the molecule is very stable and difficult to oxidise. However, by applying the hypothesis presented here, an indirect antioxidative role can be ascribed to taurine by maintaining mitochondrial oxidation and stabilising the oxidative environment and thus reduce the leakage of the reactive compounds formed inside mitochondria. On the contrary, taurine depletion will destabilise the oxidative environment with increased release of reactive oxygen species as a likely consequence. This situation will contribute to mitochondrial dysfunction.

Several clinical conditions have been reported as related to mitochondrial dysfunction, e.g., type 2 diabetes (Lowell and Shulman, 2005). Further studies are required pursuing the relationship between mitochondrial dysfunction and alterations in the cellular or mitochondrial composition like taurine depletion. However, the hypothesis presented focusing on the possible role of taurine as mitochondrial matrix buffer represents a new environmental concept for the understanding of mitochondrial dysfunction.

9. ACKNOWLEDGMENTS

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CHARACTERIZATION OF TAURINE AS INHIBITOR OF SODIUM GLUCOSE TRANSPORTER

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1. ABSTRACT

The most characterized roles of taurine include osmoregulator and membrane-stabilizing activities. However, much remains to be understood about its role in human physiology concerning its anti-hyperglycemic effect. Studies indicate that taurine-supplemented diet helps alleviate hyperglycemia or insulin resistance. This hypoglycemic effect has been postulated as taurine helping to increase the excretion of cholesterol. Alternatively, this study investigated the effect of taurine on glucose transporter using heterologous expression of sodium-glucose transporter-1 (SGLT-1). SGLT-1 was expressed in *Xenopus* oocytes and the effect of taurine on the expressed SGLT-1 was analyzed utilizing 2-deoxy-D-glucose (2-DOG) uptake and voltage clamp studies. In the oocytes expressing SGLT-1, taurine was shown to inhibit SGLT-1 activity compared to the non-treated controls in a dose-dependent manner. In the presence of taurine, the glucose uptake was greatly inhibited and the glucose-generated current was significantly inhibited. Synthetic taurine analogs were also shown to be effective in inhibiting SGLT-1 activity in a manner comparable to taurine. These effects might offer a promising opportunity in designing functional foods with anti-hyperglycemic potential by supplementing taurine and its analogs to the diet.

Key words: SGLT-1, Taurine, *Xenopus* oocytes, voltage clamp, diabetes

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2. INTRODUCTION

Taurine has been highly regarded as an effective medicine with anti-aging and osmo-regulatory function (Satsu *et al.,* 2003; Takashi *et al.,* 2004). Lowering the blood sugar content is considered one of the most effective outcomes among numerous others exerted by taurine (Hansen *et al.,* 2001; Arany *et al.,* 2004; Di Leo *et al.,* 2004). Despite many researchers' efforts to understand the basis of the hypoglycemic effect of taurine, the mechanism remains at large to date. The core of understanding the hypoglycemic mechanism lies in an analysis of glucose transport in the presence of taurine as compared to known glucose transporter inhibitors (Oulianova *et al.,* 2001).

Two lines of the glucose transporter have been identified in humans: the facilitated diffusion glucose transporters (GLUTs) and the sodium-dependent glucose transporters (SGLTs) (Doege *et al.,* 2001). Both classes of transporters are integral membrane proteins that mediate the transport of glucose and structurally related substances across membranes. Especially, SGLT-1 has an unusual affinity constant ($K_{0.5}$=0.4 mM) for glucose when compared to other SGLTs whose $K_{0.5}$ ranges 2-6 mM and which re-absorb the majority of glucose from the kidney's proximal tubule despite their small capacity. Inhibition of SGLT-1 would therefore accelerate removal of glucose from urine by blocking the re-absorption activity.

The GLUT gene encodes a protein involved in the active transport of glucose and galactose into eukaryotic and some prokaryotic cells. Seven different GLUTs have been identified to date (Ader *et al.,* 2001; Sheppers *et al.,* 2004). While the GLUTs catalyze glucose transport via a passive mechanism, the members of the SGLTs mediate active transport of glucose against its concentration gradient (Wood and Trayhun, 2003). Under certain circumstances, a low-glucose diet may circumvent the problem of glucose uptake via GLUTs. However, the glucose uptake via SGLTs continues in spite of the diet low in glucose. The human SGLT family consists of two members. The SGLT-1 transports glucose and galactose with similar affinity while the SGLT-2 highly prefers glucose to galactose. This sodium-dependent transport of D-glucose by SGLT is promoted by an inside negative membrane potential and acidity, and inhibited by phloridzin, a specific competitor of SGLT.

This study focused on the inhibition of SGLT-1 by taurine to determine its probable mechanism as a hypoglycemic agent since SGLT-1 contributes significantly to sustaining high blood glucose with its reabsorbing potential against the concentration gradient. The *Xenopus* oocyte expression system has been proven effective to study the characteristics of glucose transporters (Mandal *et al.,* 2003). Human SGLT-1 was expressed in *Xenopus laevis* oocytes and the effect of taurine and its analogs was studied using electro-physiological measurement and D-glucose influx studies.

3. MATERIALS AND METHODS

3.1. Expression of SGLT-1 cRNA in Oocytes

The cRNA for human SGLT-1 was synthesized from pSP6-hSGLT-1 with Sp6 polymerase according to the manufacturer's protocol (Promega, WI, USA). An ovary was manually removed from an adult *Xenopus* and defolliculated oocytes were injected with hSGLT-1 cRNA as described by Lee *et al.* (1998, 1995). Before microinjection, the

oocytes were washed copiously in Barth's solution [5 mM KOH, 100 mM NaOH, 0.5 mM $CaCl_2$, 2 mM $MgCl_2$, 100 mM methanesulfonic acid, and 10 mM HEPES (pH 7.4)], and stage 4 or 5 oocytes were injected with 50 nl of injection mixture containing 50 ng cRNA either of hSGLT-1 or human EAAC-1 glutamate transporter. Following injection, the oocytes were incubated in Barth's solution at 14°C for 24 h before glucose uptake and electrophysiological assays.

3.2. Analysis of hSGLT-1 Expression

After microinjection and incubation, SGLT-1 was extracted in phosphate-buffered saline (PBS) with 0.2% mercaptoethanol using a Dounce homogenizer. In other experiments, the transmembrane segment of SGLT-1 was labeled by surface biotinylation according to Lee *et al.* (1998). The injected oocytes were biotinylated with 1.0 mg/ml EZ-link-sulfo-NHS-LC-biotin (Pierce, Rockford, USA) and precipitated with Neutravidin-conjugated beads. The precipitated proteins were electrophoresed and detected by Western blotting with antiserum against hSGLT-1 (Acris Antibodies, Hiddenhausen, Germany). In addition, hSGLT-1 expression was also functionally analyzed according to the rate of entry of 2-deoxy-D-[^{3}H]glucose (2-DOG) into the oocytes as described below. Saponin (Sigma-Aldrich Biochemicals, St.Louis, USA) was used as a membrane permeability enhancer and served as a positive control for the entry of 2-DOG.

3.3. Assay of 2-Deoxy-D-[^{3}H]Glucose Uptake

Sodium-dependent glucose transport was measured according to the uptake of ^{3}H-labeled 2-deoxy-D-glucose (2-DOG) as a non-metabolized model substrate. The 2-DOG uptake was assayed by incubating 5 oocytes in 2 mM 2-[^{3}H]DOG (0.08 GBq/0.5 ml) with taurine concentrations ranging from 0 to 1 mM in 1 ml of Barth's solution. After a 10-min incubation, the oocytes were thoroughly washed with cold Barth's solution and glucose uptake was analyzed for a 30-min influx period. Entry of glucose was initiated by placing five oocytes in 1 ml of Barth's solution containing 1.0 GBq of 2-[^{3}H]DOG and cold 2-DOG at concentrations of 1 to 50 mM. During the incubation, a constant osmolality of 179.1 mOsm/l was achieved by adding a 1 M sucrose solution. The oocytes were then removed to a scintillation vial containing 0.5 ml of Barth's solution and after 2 minutes transferred to another scintillation vial. Five hundred microliters of 0.1% SDS were added to both vials and mixed by vortexing. NEN scintillation cocktail (DuPont NEN, Boston, USA) was added up to 5 ml before counting. Taurine and its three analogs were also purchased from Sigma-Aldrich Biochemicals.

3.4. Electrophysiological Experiments

The two-microelectrode voltage-clamp method was employed to measure the glucose-induced current in a rapid perfusion chamber at 22-25°C with an OC-725 voltage clamp amplifier (Warner Instrument, Hamden, USA). As a negative control, the human glutamate transporters (hEAAC-1) were expressed using *in vitro* transcribed cRNA that were prepared similarly as SGLT-1. The oocytes were perfused in a solution containing 88 NaCl, 2 KCl, 1.8 $CaCl_2$, and 10 HEPES-NaOH, pH 7.4 (in mM). For sodium-free solution, Na^+ was replaced with choline and pH was adjusted with KOH. The electrodes

were filled with 3 M KCl and the membrane potential was normally maintained at a holding potential of –50 mV. Taurine inhibition was assayed on oocytes incubated with taurine whose concentrations range from 0 to 1 mM. Data collection was performed using pCLAMP 6 software (Axon Instruments, Foster City, USA).

3.5. Kinetic Analysis of Inhibition by Taurine and Its Derivatives

Zero trans influx was analyzed using 3-OMG (0.08 GBq/0.5 ml) for a 30-min influx period. The influx of 3-OMG was initiated by incubating five oocytes in 1 ml of Barth's solution containing 1.0 GBq of 2-[^{3}H]DOG and cold 3-OMG at concentrations from 1 to 100 mM at a constant osmolarity of 179.1 mOsm/l. The oocytes were transferred to a scintillation vial containing 0.5 ml of Barth's solution. In the control experiments oocytes were injected with water under the same conditions, and the control transport rates were subtracted from the transport rates for oocytes expressing SGLT-1. Equilibrium-exchange influx assays were performed at 0 to 150 mM 3-OMG after overnight incubation at 18°C in 1 m1 of Barth's solution. Osmolality was again maintained at 179.1 mOsm/l by adding 1-M sucrose. K_m and V_{max} values were calculated using GRAPHPAD PRISM^{TM} software (GraphPad Software, San Diego, USA).

4. RESULTS

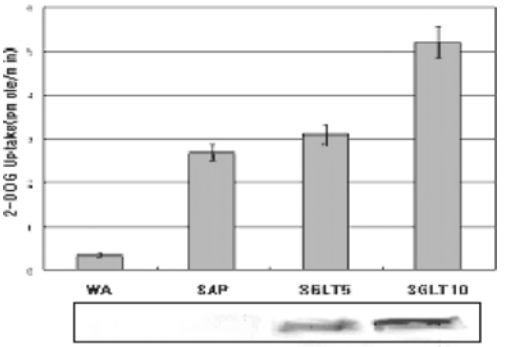

Figure 1. Functional expression of SGLT-1 in *Xenopus* oocytes. Membrane fractions were prepared from the oocytes injected with synthetic human SGLT-1 mRNA. Proteins were detected by immunoblotting using hSGLT-1. 2-DOG uptake efficiency was compared among the oocytes that were injected with water (WA) or SGLT-1 message. Following injection, the oocytes were incubated with 5-mM 2-DOG for 6 hours. The entry of 2-DOG into the oocytes was measured after copious washing with Barth's solution. SAP refers to the oocytes treated with 0.01% saponin (w/v). SGLT5 refers to the oocytes injected with 5 ng of SGLT-1 cRNA, and SGLT10 to those injected with 10 ng of SGLT-1 cRNA.

This study investigated whether taurine could serve as an inhibitor of SGLT-1. In order to understand the mechanism of the hypoglycemic effect of taurine, the extent of inhibition by taurine and its derivatives was measured in *X. laevis* oocytes expressing the SGLT-1 on their membranes. Following injection of synthetic human SGLT-1 mRNA, the expressed proteins were electrophoresed and detected by immunoblotting using anti-hSGLT1. No hSGLT-1 was detected in the oocytes injected with water and saponin

(Fig. 1). In terms of activity, the uptake of 2-DOG increased in the oocytes expressing SGLT-1, and the oocytes injected with 10 ng of the SGLT-1 mRNA took up more 2-DOG than those injected with 5 ng of mRNA. These findings are consistent with previous studies (Doege *et al.,* 2000; Olianova *et al.,* 2001).

To measure the effect of taurine on the activity of SGLT-1, voltage clamp experiments were performed. Fig. 2 shows that the oocytes expressing hSGLT-1 generated a substantial current upon addition of 100 mM D-glucose and this current was inhibited by pre-incubation with 0.5 mM phloridzin (Fig. 2A). When pre-incubated with 0.5 mM taurine (Fig. 2B), the oocytes show little or no sodium ion-induced current, similarly to the effect of phloridzin. When the oocytes were washed out with perfusion media, the electric current was induced to the level of -120 nA. When the oocytes were placed in taurine-free media, the current was restored indicating that the inhibition was reversible. Fig. 2C indicates that taurine did not affect the human Na^+/glutamate co-transporter. The oocytes expressing the human Na^+-glutamate cotransporter were incubated with 1 mM glutamate and approximately 100 nA was recorded as the peak current. When treated with 0.5 mM taurine (open band), the oocytes showed no significant alteration in the glutamate-induced currents, differently from those expressing SGLT-1. The oocytes also showed no change in the maximum glutamate-induced electric current after they had been washed with perfusion media to remove taurine. This indicates that taurine targets at SGLT-1 as a specific inhibitor.

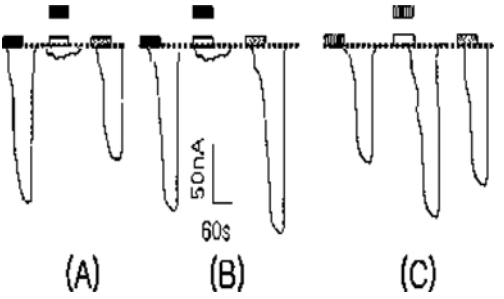

Figure 2. Voltage clamp assay on glucose-induced current in a single oocyte. An oocyte expressing hSGLT-1 was subjected to electrophysiological measurements at a membrane potential of -50 mV. (A) With the addition of 100-mM glucose (closed band), the oocytes showed -150-nA Na^+-inward current. Pre-treatment with 0.5 mM phloridzin for 1 minute (open band), however, inhibited generation of any significant current, even with the addition of 100-mM glucose. When washed out with the perfusion medium (dotted band) and insulted with 100 mM glucose, the oocytes resumed their electrical response. (B) When pre-incubated with 0.5 mM taurine (open band), the oocytes did not show any significant Na^+-induced current, similarly to the effect of phloridzin. As in Fig. 2A, the oocytes were washed with perfusion media (dotted band), and the electric current was induced to the level of -120 nA. (C) The oocytes expressing the human Na^+-glutamate cotransporter (EAAC-1) were incubated with 1 mM glutamate (striped band) and approximately 100 nA was recorded as the peak current. When treated with 0.5 mM taurine (open band), the oocytes showed no significant alteration in the glutamate-induced currents, differently from those expressing SGLT-1. The oocytes also showed no change in the maximum glutamate-induced electric current after they had been washed out with perfusion media to remove taurine (dotted band).

The inhibitory effect of taurine on SGLT-1 was analyzed in the presence of 0.5 mM 2-DOG on the basis of kinetics (Fig. 3A). The SGLT-1 activity was significantly reduced

at the fixed concentration of 2-DOG, and the fractional uptake rate (V/V_o= inhibited/non-inhibited uptake rate) declined in a dose-dependent manner. In the presence of taurine, the half-saturation rate and the maximum velocity of glucose uptake were affected in a dose dependent manner. The change in the fractional uptake value is comparable with phloridzin. Fig. 3B shows that both the apparent half-saturation rate constant and the maximum velocity of zero-trans 3-OMG uptake were decreased by taurine. The reduction in the half-saturation rate constant caused by taurine was confirmed in an equilibrium-exchange experiment using 3-OMG as a glucose analog.

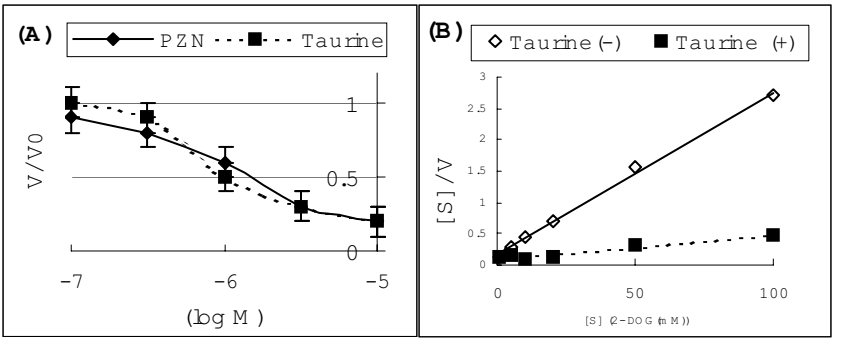

Figure 3. Effects of taurine on SGLT-1 in glucose uptake. (A) The effects of taurine on SGLT-1 were measured in the presence of 0.5 mM 2-DOG. *Xenopus* oocytes expressing SGLT-1 were incubated with taurine (square) or phloridzin (diamond). The fractional uptake rates (V/Vo) were calculated as fractional values of the inhibited (V) and non-inhibited (Vo) uptake rate. (B) Kinetic analysis on taurine inhibition of 3-OMG uptake. The zero-trans influx of 3-OMG at the indicated concentrations was determined in the oocytes expressing SGLT-1 in the absence or presence of 0.5 mM taurine (Michaelis-Menten graphs). The equilibrium exchange influx kinetics was determined at the 3-OMG equilibrium concentrations indicated. The straight line refers to the taurine-free treatment and the dotted one to the taurine treatment. The accumulation of 3-OMG was measured for 1 h and expressed as modified by logarithmic conversion. At each time point, 5 oocytes were measured per group in the assay. The negative reciprocals of the slopes were used to plot against the 3-OMG concentrations according to the Hanes plotting application. On the y-axis, the 1/[slope] refers to the reciprocal of each absolute value (taurine-free treatment: K_m=25.0 mM and V_{max}=155±12; taurine-supplemented treatment: K_m=4.9±1.1 and V_{max}=28±6).

Unlike 2-DOG, 3-OMG is transported by SGLT-1 but not phosphorylated following its entry into the cell. At an equilibrium concentration of 100 mM 3-OMG, the rate of accumulation of 3-OMG was severely impaired by taurine. The Michaelis-Menten constants of SGLT-1 obtained in these equilibrium exchange and zero-trans influx experiments are consistent with previous reports. Taurine analogs also inhibited SGLT-1 activity (Fig. 4), hypotaurine being the most effective. This result suggests that the main skeleton of taurine functions in the inhibition of SGLT-1 and the extent of inhibition can be upgraded by changing functional groups. Screening of a chemical library could also identify even more effective taurine-based inhibitors.

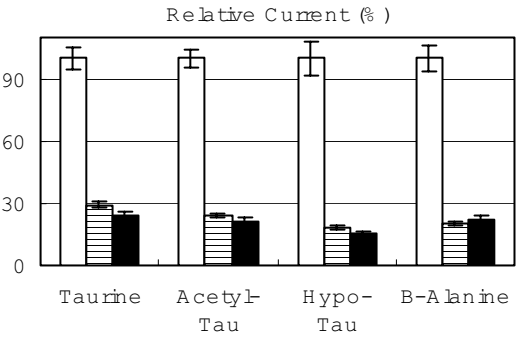

Figure 4. Effect of taurine analogs on SGLT-1. Effect of taurine analogs on SGLT-1 was assayed using voltage clamp methods. The effects of taurine analogs on SGLT-1 were measured in the presence of 50 mM glucose. The measurements were expressed as relative values with the taurine-free value as 100%. Each column represents the mean ± S.E. (n=5). The open bars refer to non-treated controls. The striated and dark ones refer to the treatment of 1 mM of each taurine analog and phloridzin, respectively. B-alanine: beta-alanine.

5. DISCUSSION

The present study shows that taurine inhibits glucose transport by SGLT-1. Throughout this study, taurine consistently showed a potent inhibitory effect on the transport of glucose governed by SGLT-1. This study also indicates that taurine is a promising natural source of biologically active substances to cope with hyperglycemia. Taurine significantly decreased the inward transport of 2-DOG in *Xenopus laevis* oocytes expressing SGLT-1, thus underlining the potential importance of taurine and its derivatives as SGLT inhibitors. In treating diabetes, taurine should provide important alternative routes for reducing glucose levels as a specific inhibitor for SGLT. The mode of inhibition of SGLT by taurine appears to be highly specific: Lack of the inhibitory effect on EAAC-1 (sodium-glutamate transporter) expressed in the oocytes indicates that taurine serves as an actual inhibitor against SGLT-1.

In the kinetic analysis, taurine significantly lowered both K_m and V_{max} of SGLT-1 in a dose-dependent manner. Its mode of action also resembled that of phloridzin, a known inhibitor of SGLT-1. This reduction was most likely due to a decreased affinity for the transported substrate considering that the K_m was affected. Thus, it is likely that taurine affects one or more steps in transport at the point of substrate binding. Taurine also caused a more than sixfold reduction in the apparent affinity of SGLT-1 for 3-OMG. The kinetic analysis also indicated that taurine affects the equilibrium binding of glucose to the glucose transporter. This provides good evidence that taurine acts directly on SGLT-1 rather than affecting a signaling pathway leading to glucose uptake and metabolism.

Taurine analogs also have potential as inhibitors of SGLT-1 despite the fact that their chemical structure differs from that of the common SGLT inhibitors. Taurine analogs inhibited glucose influx via hSGLT-1, hypotaurine giving the highest level of inhibition. These compounds can easily be synthesized in quantity, and are already listed in many

commercial catalogues. Thus these compounds would therefore be readily used as potential agents to treat the problem of high blood glucose levels, especially to prevent postprandial hyperglycemia. To date, the available specific non-transportable inhibitors of SGLT-1 are glucosides of flavonoid-like polyphenols such as phloridzin. The steps in glucose transport targeted by these glucosides are well known. Since taurine differs from those glucoside and its analogs, the inhibition of SGLT-1 by taurine implies that additional steps affecting the overall performance of SGLTs might exist to be further elucidated.

In the present study, taurine was tested for the hypoglycemic effect at the concentration ranging from 0.2 to 1.0 mM. These limits were adopted because phloridzin, the known SGLT-1 inhibitor, has been usually applied in this concentration range. It is undertaking to determine the working level of taurine as an anti-hyperglycemic agent in the context of physiological ramification to humans. Since this study was performed using the *Xenopus* oocyte systems, further study must be performed to investigate which *in vivo* level of taurine is relevant to treat hyperglycemia.

In conclusion, the results of this study have shown that taurine can inhibit glucose transport through SGLT-1. This inhibitory effect cannot be attributed to an indiscriminative effect on transmembrane proteins. The inhibitory effect of taurine does not lie at the level of glucose metabolism; most likely taurine inhibits glucose transport via SGLT-1. The basis for the inhibitory effect of taurine relies on the step of translocation through glucose transporters. Finally, taurine can be used to treat diabetes and has a specific inhibitory effect on SGLT-1.

6. ACKNOWLEDGMENTS

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TAURINE ATTENUATES PYRIDOXAL-INDUCED ADRENOMEDULLARY CATECHOLAMINE RELEASE AND GLYCOGENOLYSIS IN THE RAT

Jagir P. Patel and Cesar A. Lau-Cam*

1. ABSTRACT

The vitamin B_6 vitamers, pyridoxine, pyridoxal and pyridoxamine are capable of promoting the mobilization of hepatic glycogen stores and hyperglycemia in the rat. These effects, which are dose-related and far greater with pyridoxal than with the other B_6 vitamers, follow the outpouring of adrenomedullary catecholamines into the circulation. By taking advantage of this animal model, the present study was undertaken to examine the validity of a previously held view that taurine can suppress the release of adrenomedullary catecholamines. By treating Sprague-Dawley rats with intraperitoneal doses of pyridoxal, taurine, β-alanine, specific pharmacological antagonists (atropine, hexamethonium, labetalol, propranolol, verapamil) and their combinations, it was determined that the attenuating action of taurine on pyridoxal-induced glycogenolysis is centered in the adrenal gland.

2. INTRODUCTION

Among the myriad of biological actions manifested by taurine (TAU) in mammalian organisms and test preparations, its role in preventing the release of catecholamines (CATs) from the adrenal gland is probably one of the least studied. In fact, the only published reports on this subject appears to have come from work by Nakagawa and Kuriyama (1975) and Kuriyama and Nakagawa (1976) describing the effects of orally administered taurine on alterations in adrenal function induced by immobilized cold stress in the rat, and manifested by a reduction in epinephrine content of the adrenal medulla and an increase in blood glucose. On the other hand, work in this laboratory on the biological actions of the vitamin B_6 vitamers pyridoxal (PL), pyridoxine (PN), and pyridoxamine (PA) in the rat has shown that these pyridine

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derivatives are endowed with the ability to stimulate the release of adrenomedullary catecholamines (CATs) in a dose-dependent manner when administered by the oral or intraperitoneal routes and at doses above 100 mg/kg (Kendall, 1984; Lau-Cam *et al*., 1991). The outflow of CATs into the circulation is found to be followed almost immediately by an extensive mobilization of the hepatic glycogen and a marked rise in plasma glucose.

The objectives of the present study were first, to determine in the rat whether TAU can attenuate the release of adrenomedullary CATs elicited by chemical stimulation with a compound such as vitamin B_6, and second, to gain information on the mechanism leading to the release of CATs and the decrease in hepatic glycogen. To this effect, groups of fasted rats were separately treated with PL, TAU, β-alanine (BALA), a pharmacological antagonist (adrenoceptor, muscarinic, ganglionic, calcium channel) and combinations thereof, and plasma CATs, hepatic glycogen and plasma glucose were measured at predetermined times.

3. MATERIALS AND METHODS

3.1. Animals

All experiments were conducted on groups of 5 male Sprague-Dawley rats, 225-250 g in weight, purchased from Taconic (Germantown, NY) and housed in groups of 5 in plastic cages, in a room maintained at a constant temperature of 21±3˚C, a constant humidity and a normal 12 h light-dark cycle. During an acclimation period of at least 3 days, the rats were fed a commercial rat diet (Purina™ Lab Chow, Ralston-Purina Co., St. Louis, MO) and water ad libitum. The food was removed 12 h before an experiment.

3.2. Treatments

The various test compounds were administered as aqueous solutions, by the intraperitoneal route, and at a dose of 300 mg/kg (PL hydrochloride, PN, PA hydrochloride), 2.4 mmol/kg in equal divided doses (BALA, TAU), 2 mg/kg (labetalol hydrochloride = LAB, propranolol hydrochloride = PRO, phentolamine hydrochloride = PHA, verapamil hydrochloride = VER, 28 mg/kg (hexamethonium bromide = HEX) or 0.02 mg/kg (atropine sulfate = ATR). Each compound was administered 30 min before PL. When BALA or any of the pharmacological antagonists was added to TAU, they preceded the first dose of TAU by 30 min. Divided doses were administered 30 min apart and 30 min before PL.

3.3. Samples and Assays

Blood samples were collected at 0, 5, 10, 15 and 30 min post-PL by the orbital sinus technique (Riley, 1960) into microtubes containing a small amount of NaF-Na_2EDTA, mixed well, and centrifuged at 2,500 rpm for 10 min to isolate the plasma fraction. The samples were kept on ice, pending their analysis for glucose levels, or frozen, until analyzed for CATs. Plasma glucose was measured using a commercially available kit (Procedure No. 510 from Sigma Chemical Co., St. Louis, MO). Plasma CATs (epinephrine = E, norepinephrine = NE, dopamine = DA) were measured by the

HPLC method with electrochemical detection described by Williams *et al*. (1985), after a sample cleanup by the method of Wang *et al*. (1999), and using 3,4-dihydroxy-benzylamine as an internal standard. Livers were removed by the freeze-clamp technique of Wollenberger *et al*. (1960) immediately after collecting the 30 min blood sample, and kept at -70˚C until their assay for hepatic glycogen according to Keppler and Decker (1974). A sample for this purpose was prepared by homogenizing a portion of frozen liver with ice-cold 0.6 M $HClO_4$ (1 g/5 ml), neutralization of an aliquot of the homogenate (0.2 ml) with 1 M $KHCO_3$ (0.1 ml), and hydrolysis by incubation (40˚C) with a solution of amyloglucosidase containing 45 units/ml (2 ml). Following centrifugation of the suspension, the free glucose present in the supernatant was measured as described for the plasma samples. The concentrations of the various analytes were calculated from the experimental values obtained for the corresponding standard preparations, treated in identical manner as the samples.

3.4. Statistical Analysis

The experimental results are reported as the mean ± SEM for n = 5. They were analyzed using a commercially available software program (SPSS™ Version 12.0, John Wiley & Sons, New York, NY). Differences were considered to be statistically significant at $p<0.05$ by unpaired Student's t-test and one-way ANOVA followed by Neumann-Keuls *post-hoc* test.

4. RESULTS

Initially, PN, PL and PA were compared with one another in terms of their effects in releasing adrenal CATs, mobilizing the hepatic glycogen, and elevating the circulating levels of glucose using a dose (300 mg/kg) that had been previously determined not to be lethal or overly convulsant. In this manner, the potency of the three vitamin B_6 vitamers was found to differ from compound to compound, Whereas PN and PA demonstrated minimal effects, PL increased the plasma CAT by about 71%, lowered the hepatic glycogen by about 11%, and raised the plasma glucose by about 8% (Figs. 1-3). Any salient differences among the three B_6 vitamers was obviated either by interruption of the splanchnic innervation to the adrenal gland by splanchnectomy or by bilateral adrenalectomy (Figs. 1-3). Based on these results, all subsequent experiments were conducted only with PL.

The results shown in Table 1 indicate that the actions of PL are dose-dependent in the concentration range 75-450 mg/kg, with the effects becoming significantly different from the control group ($p<0.001$) at a dose above 75 mg/kg. Elevations in plasma total CATS ranged from 5% to 213% in direct proportion to the dose of PL used. In all instances, the CATs were released in the concentration order E>NE>>DA. Changes in plasma glucose were noticeable as early as 5 min post-PL and continued to rise for at least the next 25 min. The elevations in plasma glucose and decreases in hepatic glycogens levels at 30 min post-PL ranged from 15% to 337% and from 1.5% to 22%, respectively.

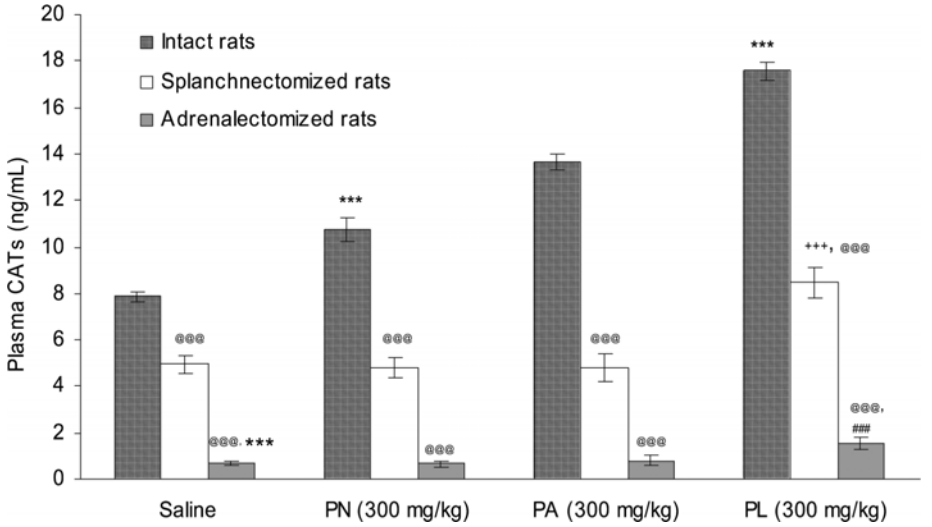

Figure 1. Effect of PN, PA and PL on the plasma CATs of intact, splanchnectomized and adrenalectomized rats. Bars represent the mean for 5 rats. Vertical lines represent the SEM. Significance *vs.* intact rats (saline): ***p<0.001; *vs*. splanchnectomized rats (saline): $^{+++}$ p<0.001; *vs*. adrenalectomized rats (saline): $^{\#\#\#}$ p<0.001; *vs*. intact rats; $^{@@@}$ p<0.001.

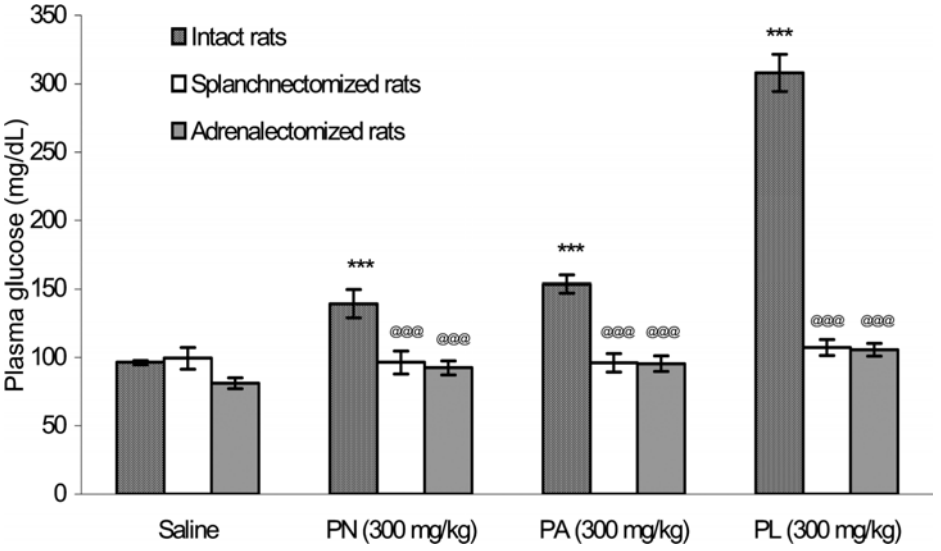

Figure 2. Effect of PN, PA and PL on the plasma glucose of intact, splanchnectomized and adrenalectomized rats. Bars represent the mean for 5 rats. Vertical lines represent the SEM. Significance *vs*. intact rats (saline): ***p<0.001; *vs*. intact rats: $^{@@@}$ p<0.001.

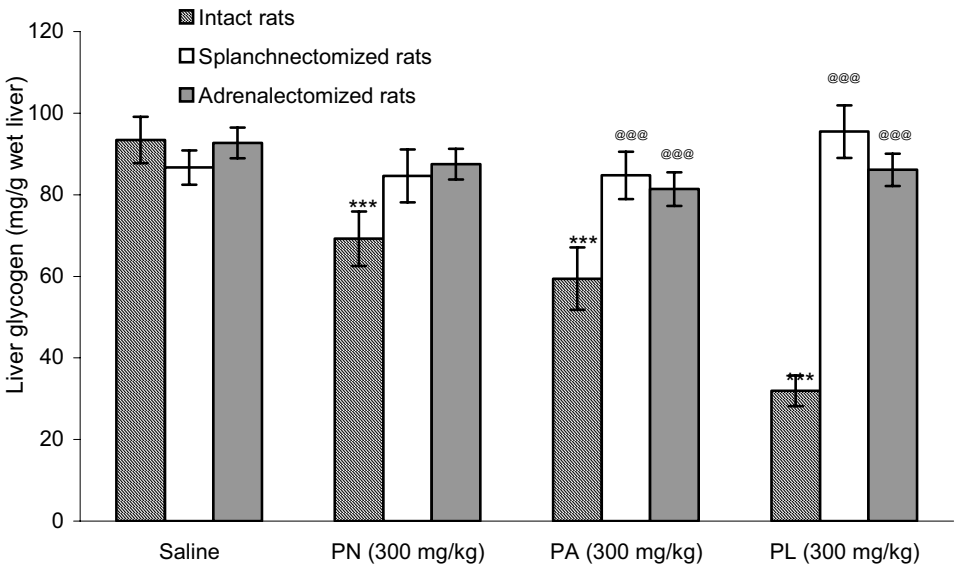

Figure 3. Effect of PN, PA, and PL on the liver glycogen in intact, splanchnectomized and adrealectomized rats. The bars represent the mean of 5 rats. The vertical lines represent SEM. Significance *vs.* inracr rats: ***p<0.001; *vs.* splenectomized rats (saline): $^{+++}$p<0.001; *vs.* intact rats: $^{@@@}$p<0.001.

Table 1. Effect of different doses of PL (75-450 mg/kg) on the plasma total CATs, plasma glucose and liver glycogen 30 min after a treatment with PL (300 mg/kg)[a,b]

Treatment (mg/kg)	Plasma total CATS (ng/ml)	Plasma glucose (mg/dl)	Liver glycogen (mg/g wet liver)
Saline	7.89 ± 0.08	96.35 ± 1.43***	93.40 ± 5.69
PL (75)	8.29 ± 0.07	110.64 ± 3.01	92.03 ± 4.52
PL (150)	10.75 ± 0.09***	158.00 ± 3.07***	58.52 ± 5.86***
PL (300)	17.56 ± 0.41***	307.87 ± 3.58***	31.94 ± 3.79***
PL (450)	24.68 ± 0.30***	420.57 ± 9.06***	20.29 ± 5.83***

[a] Results are given as the mean ± SEM for groups of 5 rats each. [b]Statistical comparisons: ***p<0.001 vs. saline.

Pretreating rats with TAU (1.2 or 2.4 mmol/kg) attenuated all of the biochemical alterations brought about by a single dose (300 mg/kg) of PL (300 mg/kg). Furthermore, the administration of the higher dose of TAU in two portions (i.e., 1.2 mmol/kg + 1.2 mmol/kg) led to a better protection than an equipotent (i.e., 2.4 mmol/kg) bolus dose (Table 2). Thus, a 2.4 mmol/kg of TAU lowered the plasma total CATs seen with PL alone by about 30% (p<0.001) when given at once, and by 42% (p<0.001) when given in divided doses. Likewise, TAU attenuated the elevations in plasma glucose induced by PL from 5 min and 2.4 mmol/kg as a single dose, and by

Table 2. Effect of the dose of TAU (1.2 mmol/kg, 2.4 and 1.2 + 1.2 mmol/kg) on the plasma total CATs, plasma glucose and liver glycogen 30 min after a treatment with PL (300 mg/kg)[a,b]

Treatment (mg/kg)	Plasma total CATS (ng/ml)	Plasma glucose (mg/dl)	Liver glycogen (mg/g wet liver)
Saline	7.89 ± 0.08***	96.35 ± 1.43***	93.40 ± 5.69***
PL (300)	17.56 ± 0.41	307.87 ± 3.58	31.94 ± 3.79
TAU (1.2) + PL (300)	14.95 ± 1.01***	185.84 ± 7.86***	47.64 ± 4.69***
TAU (2.4) + PL (300)	12.10 ± 0.08***	166.71 ± 4.64***	57.96 ± 1.72***
TAU (1.2 + 1.2)	8.24 ± 0.15***	106.12 ± 2.54***	92.83 ± 6.72***
TAU (1.2 + 1.2) + PL (300)	10.20 ± 0.06***,###	149.53 ± 3.25***,###	65.21 ± 6.90***,###

[a] Results are given as the mean ± SEM for groups of 5 rats each. [b] Statistical comparisons: ***p<0.001 *vs*. PL; ###p<0.001 *vs*. TAU +.

51% with 2.4 mmol/kg in divided doses, respectively, p<0.001 for all treatments). The corresponding increases in hepatic gloycogen were 49%, 81.5% and 104%, respectively, relative to PL-alone (p<0.001 for all treatments).

The need for TAU to be taken up into target cells to provide its protective action was studied using BALA, a known competitive inhibitor of TAU transport (Shaffer and Kocsis, 1981) and depleter of intracellular TAU (Waterfield *et al*., 1993). Even though BALA decreased the protective action of TAU against PL-related biochemical alterations, thus suggesting a decrease in TAU cellular uptake, a straightforward interpretation of the present results is not possible since BALA manifested altering actions of its own (Table 3). For example, by itself BALA induced a 43% increase in plasma CATs and, when given before PL or TAU plus PL, it attenuated the PL-induced elevation by about 26% and the protection offered by TAU by about 15%, respectively (Table 3). Similar trends emerged from the results for the plasma glucose and hepatic glycogen levels. In the presence of BALA the increase in plasma glucose seen with TAU plus PL alone rose from 55% to 144%, but that seen with PL alone was reduced from 220% to 193%. Similarly, hepatic glycogen losses by TAU plus PL were greater in the presence (51%) than in the absence (30%) of BALA. By itself BALA increased the plasma glucose (15%, p<0.001) and had a negligible decreasing effect (~2%) on the baseline hepatic glycogen (Table 3).

The cholinergic component of catecholamine release was studied by blocking nicotinic and muscarinic acetylcholine receptors with HEX and ATR, respectively. The results of these studies are presented in Table 3. HEX drastically reduced the elevation in plasma total CATs (by 51%) and plasma glucose (by 56%) and elevated the liver glycogen (by 123%) relative to PL alone. Combining HEX with TAU led to a small enhancement of the effect of HEX on the elevation of the plasma total CAT (55% decrease) and plasma glucose (64% decrease) and on the liver glycogen retention (155% increase). In comparison to HEX, antagonism of PL-mediated effects by ATR was, at best, modest. Thus ATR reduced the rise in plasma total CAT by only 5% and in plasma glucose by 10%. On the other hand, a gain of only a 2% in glycogen retention

was observed with ATR pretreatment. Although combining ATR with HEX did not alter the effect of HEX alone on the plasma total CATs and glucose levels induced by PL, it, however, enhanced the retention of liver glycogen by an additional 16% (i.e., 171% vs. 155%). Because of the weak effects of ATR on PL-induced biochemical alterations, this agent was not tested further as a combination with TAU.

Table 3. Effect of TAU, BALA, ATR, HEX, VER, PRO, LAB, PHA and their combinations on the plasma total CATs, plasma glucose and liver glycogen 30 min after a treatment with PL (300 mg/kg)[a,b,c]

Treatment	Plasma total CATs (ng/ml)	Plasma glucose (mg/ml)	Liver glycogen (mg/g wet liver)
Saline	7.89 ± 0,22***	96.35 ± 1.43***	93.40 ± 5,69*
PL	17.56 ± 0.41	307.87 ± 13.58	31.94 ± 3,79
TAU	8.24 ± 0.15***	106.12 ± 2.54***	92.83 ± 6.72***
TAU + PL	10.20 ± 0.06***	149.53 ± 3.25***	65.21 ± 6.90***
BALA	11.26 ± 0.18***	110.54 ± 4.58***	91.72 ± 6.30***
BALA + TAU	9.60 ± 0.24***	282.49 ± 4.01**	34.86 ± 2,69
BALA + PL	15.52 ± 0.13***	97.15 ± 1.49***	93.79 ± 4.79***
BALA + TAU + PL	11.30 ± 0.25***	235.03 ± 5.99***,###	45.46 ± 3.21***,###
ATR + PL	16.69 ± 0.19***	277.24 ± 7.19*,###	32.46 ± 3.10###
HEX + PL	8.63 ± 0.16***	135.76 ± 3.73***,###	71.29 ± 5.28***
HEX + TAU + PL	7.93 ± 0.02***	111.73 ± 4.97***,###	81.43 ± 6.39***
ATR + HEX + PL	8.01 ± 0.21***	117.19 ± 3.49***	86.55 ± 4.76***
VER + PL	16.35 ± 0.21***	208.71 ± 3.35***	55.67 ± 2.91***
VER + TAU + PL	10.23 ± 0.18***	136.18 ± 0.76***,###	84.33 ± 3.54***,###
PRO + PL	20.00 ± 0.07***	133.30 ± 3.10***	93.71 ± 5.42***
PRO + TAU + PL	10.57 ± 0.24***	81.05 ± 2.39***,###	100.71 ± 4.02***,###
LAB + PL	19.28 ± 0.39	134.00 ± 2.10***	92.81 ± 4.36***
LAB + TAU + PL	10.34 ± 0.39***	85.11 ± 3.19***,###	97.04 ± 3.54***,###
PHA + PL	18.65 ± 0.36	272.57 ± 5.06**	35.75 ± 2.73
PHA + TAU + PL	10.77 ± 0.09***	81.05 ± 2.39***,###	65.65 ± 4.30***

[a] Treatment doses are given in the experimental section. [b] Results are given as the mean ± SEM for groups of 5 rats each. [c] Statistical comparisons: *p<0.05, **p<0.01, ***p<0.001 vs. PL; ###p<0.001 vs. TAU+PL.

Since voltage-sensitive calcium channels located on the chromaffin cells are responsible for the release of CATs from adrenal glands upon nerve stimulation, a calcium channel antagonist such as VER was used as a pretreatment for PL. Although VER reduced the release of CATs (by <8%) its effects was only modest when compared to that of HEX and, when given together with TAU it did not supplement the action of TAU on PL to any extent As a result, VER did not protect against either the rise in plasma glucose or the loss of hepatic glycogen induced by PL (Table 3).

To ascertain the contributions of hepatic adrenoceptors to PL-mediated glycogenolysis, rats were separately treated with a nonselective β-adrenoceptor blocker (PRO), an α- and β-adrenoceptor blocker (LAB), and an α_2-adrenoceptor blocker (PHA) in conjunction with PL or with TAU plus PL. The results of these experiments

are summarized in Table 3. None of the adrenoceptor blockers were able to attenuate the actions of PL on the adrenal gland or to enhance the potency of TAU on PL-related release of adrenomedullary CATs to a significant extent. In contrast, PRO and LAB, but not PHA, were able to magnify the antagonistic action of TAU toward the hyperglycemia and depletion of hepatic glycogen that followed an acute high dose of PL. In this context, a combined treatment with either PRO and TAU or LAB and TAU prior to PL preserved the hepatic glycogen to levels comparable to those of the control group and reduced the elevation of plasma glucose due to PL by 74% and 72%, respectively. Moreover, these plasma glucose values were 16% and 12% below those of control values, respectively. Under the same experimental conditions, PHA was without effect against PL.

5. DISCUSSION

In the rat, the reported intravenous and oral median lethal doses for PN range respectively from 530 to 657 mg/kg and from 4.0 to 5.5 g/kg (Unna and Honig, 1968), with tonic convulsions preceding death in each case. In humans and laboratory animals, megadoses of PN equal to 100-200 times a recommended daily allowance (RDA) of 1-4 mg/day (Brubacher, 1989) are known to cause, after a 2 to 4 months intake, neurotoxicity characterized by temporary peripheral motor and sensory neuropathy, loss of limb reflexes, unsteady gait, the loss of sensory perception in the limbs, and nonspecific axonal degeneration of large and small myelinated fibers in peripheral sensory nerves (Schaumburg *et al*., 1983). While PN and PA share similar toxicity and safety profiles, PL is found to be at least twice as potent as either PN or PA (Kraft *et al*., 1961). In the present study, by using an intraperitoneal dose of a vitamin B_6 vitamer that lies at the threshold above which frank toxicity and lethality can develop (i.e., 300 mg/kg), it was possible to verify that the extent of the biochemical alterations caused by each B_6 vitamer follow the same order as their intrinsic toxicity, namely PL>PA>PN.

On the basis of the results of earlier work from this laboratory in which bilateral adrenalectomy and splanchnectomy were shown to dramatically reduce the stimulatory effect of the various vitamin B_6 vitamers on hepatic glycogenolysis and to make their effects equivalent with one another, a common mechanism of action for the three B_6 vitamers (Lau-Cam *et al*., 1991) is inferred. In this context, to confirm the existence of a mechanism that entails central neural stimulation of the sympathetic branch of the autonomic nervous system and culminates in the release of CATs from the chromaffin cells of the adrenal medulla, pharmacological blockers such as ATR, HEX and VER were administered prior to PL or TAU plus PL. Likewise, to more clearly define the site of action of TAU on CAT release by PL, adrenoceptopr blockers such as PRO, LAB and PHA were given as pretreatments to PL alone or to TAU plus PL.

The probable site of action of TAU on PL-related biochemical alterations was established by administering this compound in the presence and absence of pharmacological blockers relevant to glycogenolysis. Whereas most pharmacological blockers but ATR and PHA attenuated PL-related glycogenolysis and subsequent hyperglycemia to various extents (LAB, PRO>HEX>VER), all were protective when combined with TAU. The greater protection attained with LAB or PRO plus TAU than with any compound alone and the much weaker action exerted by PHA are indicative of a predominant contribution of hepatic β-adrenoceptors over α-adrenoceptors in the

mobilization of the hepatic glycogen by PL. Likewise, the contrasting effects of HEX and ATR on the plasma CATs elevation elicited by PL, being greater with the former and lesser with the latter, point to a central stimulation by PL that is transmitted to the adrenal gland by the sympathetic innervation running through the celiac ganglion and acting predominantly on nicotinic acetylcholine receptors rather than on muscarinic acetylcholine receptors. In this regard, although ATR exerted no modifying effect on the suppression of PL-induced secretion of CATs, it enhanced the attenuating action of HEX on the rise in plasma glucose and loss of hepatic glycogen by PL. A role for both types of cholinergic receptors has been demonstrated in isolated adrenal glands of spontaneously hypertensive and normotensive rats (Lim *et al*, 2002). When considered together, these results strongly suggest that the actions of TAU against the rise in plasma CATs and depletion of liver glycogen brought about by PL are limited to the adrenal gland.

According to Nakagawa and Kuriyama (1975) and Kuriyama and Nakawaga (1976), the daily oral administration of TAU to rats (4-7 g/day for 3 days) will prevent the stress-induced decline of epinephrine in adrenal medullary granules without affecting the synthesis of the amine. The regulatory mechanism proposed by these authors would involve the reduction in CAT output from the adrenal gland as a result of a stabilizing effect by TAU on the membrane of the granular membrane. On the other hand, Muramatsu *et al*. (1978) have suggested that the stabilizing effect of TAU on excitable neuronal membranes may be derived from a modulating role the release of acetylcholine and norepinephrine from neuronal tissues. Alternatively, TAU may increase the affinity of calcium for the medullary granular membrane and/or may decrease the release of calcium from the granules, situations that will lower CAT outflow from the adrenal granules.

6. CONCLUSIONS

The systemic administration of the vitamin B_6 vitamers PL, PA and PN to rats in doses at least 150-fold the median RDA of the vitamin led to an almost immediate release of adrenal CATs into the circulation, hepatic glycogenolysis, and hyperglycemia. The order of potency of the vitamers was shown to be PL>PA>PN. A pretreatment with TAU or, better, with PRO-TAU or HEX-TAU resulted in almost complete attenuation of CATs secretion and, hence, in extensive preservation of the hepatic glycogen. The present results support an effect of TAU that is centered in the adrenal component of the sympathoadrenal apparatus responsible for CAT release due to stress or chemical challenge.

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CYTOTOXICITY OF TAURINE METABOLITES DEPENDS ON THE CELL TYPE

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1. INTRODUCTION

Taurine, the most abundant free amino acid in mammalian tissues and in the cytosol of leukocytes is the major scavenger for highly reactive and toxic oxidants: hypochlorous (HOCl) and hypobromous (HOBr) acids generated by activated neutrophils and eosinophils, respectively (Thomas *et al.,* 1995; van Dalen and Kettle, 2001; Schuller-Levis and Park, 2003). Detoxification of HOCl and HOBr by taurine with resulting formation of less reactive and toxic but more stable chloramines (taurine chloramine, Tau-Cl, and taurine bromamine, Tau-Br) is thought to contribute to the protective effects of this amino acid against inflammation associated tissue damage. Accumulating evidence points out that Tau-Cl is an important physiologic effector molecule endowed with the ability to inhibit synthesis of various pro-inflammatory mediators in activated cells that participate in the inflammatory response (e.g. leukocytes, fibroblasts, dendritic cells) (Marcinkiewicz *et al.,* 1999; Chorazy *et al.,* 2002; Kontny *et al.,* 2003b; Schuller-Levis and Park, 2003). Recent data suggest that at least some of these anti-inflammatory activities can be attributed also to Tau-Br (Olszanecki and Marcinkiewicz, 2004; Marcinkiewicz *et al.,* 2005). However, we observed that, due to cytotoxicity, the range of Tau-Br anti-inflammatory activities seems to be more limited than that of Tau-Cl (manuscript in preparation). Although Tau-Cl is a rather stable oxidant, it is slowly decomposed to sulphoacetaldehyde (Cunningham *et al.,* 1998) that at physiologically relevant concentrations neither affects cell viability nor inhibits synthesis of pro-inflammatory mediators (Kontny *et al.,* 2003a).

Rheumatoid arthritis (RA) is a chronic inflammatory disorder driven by a network of interdependent pathogenic mechanisms involving innate and adaptive immunity that lead to progressive destruction of joint cartilage and bone with resulting disability (Firestein, 2003). The cells of mesenchymal origin located in the joint intimal lining named

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Taurine 6
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fibroblast-like synoviocytes (FLS) participate in RA hallmarks: chronic synovitis, synovial membrane hyperplasia and tissue destruction (Firestein, 1996). We have previously reported that at non-cytotoxic concentrations Tau-Cl inhibits *in vitro* pro-inflammatory activities of RA FLS, e.g. synthesis of IL-6, IL-8 (Kontny *et al.,* 1999a, 2000) and prostaglandin E_2 (Kontny *et al.,* 2003c) as well as proliferation of these cells (Kontny, *et al.,* 1999a), while *in vivo* it delays the onset of collagen-induced arthritis in mice (Kwasny-Krochin *et al.,* 2002). Recently, we have found that in RA FLS Tau-Cl triggers nuclear accumulation of transcriptionally active p53 tumor suppressor protein (Kontny *et al.,* 2005). Other studies revealed that HOCl acts in a dose-dependent manner and is able to trigger either growth arrest, apoptosis or necrosis of various types of cells (Vissers *et al.,* 1999; Vile *et al.,* 2000; Englert and Shacter, 2002). Moreover, some of these effects (i.e. growth arrest) were dependent on p53 activity (Vile, *et al.,* 2000) and were mediated by chloramines (Vile, *et al.,* 2000; Englert and Shacter, 2002).

We hypothesized that Tau-Cl triggers cell death via a p53-dependent pathway. This was investigated in two types of cells: (i) RA FLS, showing some preneoplastic features and responding to Tau-Cl with accumulation of p53, and (ii) leukemic human T cell line, Jurkat, known to express mutated p53 (Cheng and Haas, 1990).

2. MATERIALS AND METHODS

2.1. Cell Cultures and Treatment

Synovial tissues were obtained from the knee joints of RA patients at the time of total joint replacement surgery or synovectomy performed as a normal part of clinical care. The study was approved by the local Ethics Committee. FLS were isolated and cultured as described previously (Kontny *et al.,* 1999a). Human peripheral blood T cell leukemia line Jurkat was maintained in RPMI 1640 medium supplemented with 10% of fetal calf serum, 2 mM L-glutamine, and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml gentamycin) at 37°C with 5% CO_2. The cells were cultured in medium (control) or were treated with N-chlorotaurine sodium salt (Tau-Cl) (Gottardi and Nagl, 2002), added at 0.2–1 mM concentrations either alone or together with 1 μg/ml of monoclonal anti-human CD95/Fas antibody (R&D Systems, UK).

2.2. Cell Viability Assays

Cytotoxicity of tested compounds was evaluated after 24-72 hours of cell treatment, by measurement of lactate dehydrogenase (LDH) activity in culture supernatants using LDH assay kit (Takara Shuzo Co., Otsu, Shiga, Japan). After 4-24 hours of cell treatment, apoptosis was evaluated by measuring annexin V binding to externalized phosphatidylserine and necrosis by the permeability of cells to propidium iodide (PI) using Annexin-V-Fluos staining kit (Roche Diagnostics GmbH, Germany), followed by flow cytometric analysis. The number of early apoptotic (annexin V positive), late apoptotic (both annexin V and PI positive) and necrotic cells (PI positive) was calculated.

2.3. Measurement of Caspase Activity

After 2-8 hours treatment of the cells as above, the cell lysates were prepared and tested for the enzymatic activity of caspases, using colorimetric assays (R&D Systems, UK), based on the application of caspase 9-specific (LEHD) or caspase 3-specific (DEVD) substrate peptides conjugated to the color reporter molecule. At the same time, the expression of caspase 3 in its inactive (proenzyme) and enzymatically active (processed) forms was evaluated in the total protein fraction using rabbit anti-caspase 3 polyclonal antibody (Alexis Biochemicals, Switzerland) and immunoblotting. The same technique was applied to evaluate degradation of poly (ADP-ribose) polymerase (PARP) using monoclonal anti-PARP antibody (Sigma, St. Louis, MO, USA).

2.4. Evaluation of P53, Bax, Bcl-2 and Cathepsin D Expression

Expression of Bax and Bcl-2 proteins was estimated in the cytosolic, membrane and nuclear protein fractions prepared as described previously (Kontny *et al.,* 1999b*,* 2000). Expression of p53 protein and lysosomal aspartic protease, cathepsin D (CTSD) was evaluated in the nuclear and cytosolic fraction, respectively. After normalization of protein contents, the samples were analysed by Western blotting using primary monoclonal antibodies, peroxidase conjugated anti-mouse immunoglobulin and the chemiluminescence reagents (SuperSignal West Femto Maximum Sensitivity Substrate, Pierce Biotechnology, Rockford, IL, USA). The primary monoclonal antibodies: anti-Bax, anti-Bcl-2 and anti-cathepsin D were from Sigma, while anti-p53, recognizing both wild type and mutated p53 protein, was from Oncogene, San Diego, CA, USA. The bands corresponding to analyzed proteins were densitometrically scanned using Kodak ID Image Analysis Software (Eastmond Kodak, Rochester, NY, USA).

2.5. Statistical Analysis

The repeated-measures analysis of variance (ANOVA), followed by Tukey's test, was applied to evaluate the effects of Tau-Cl and anti-Fas treatments. The results are expressed as the mean ± SEM. P values less than 0.05 were considered significant.

3. RESULTS

3.1. Rheumatoid Arthritis Fibroblast-Like Synoviocytes

3.1.1. Tau-Cl Shifts Anti-Fas-Triggered Apoptosis Toward Necrotic Type

Treatment of RA FLS for 24–72 hours with the 200-μM or 300-μM concentration of Tau-Cl did not affect cell viability (Table 1 and Fig. 1). At the 400-μM concentration Tau-Cl was slightly cytotoxic (Table 1), but this was not accompanied by a significant elevation of either necrotic or apoptotic cell number (Fig. 1). At the 500-μM concentration Tau-Cl exerted a mild, progressive cytotoxic effect, resulting in death of 26.6± 3.5 % cells at 72 hours (Table 1). At an earlier time point (24 hours), 500 μM Tau-Cl mildly elevated the number of necrotic cells (6.8 ±0.5%) (Fig. 1). In the presence of 1 mM Tau-Cl there was a marked increase of both late apoptotic and necrotic cell number,

Table 1. Cytotoxic effects of Tau-Cl and anti-Fas antibody on RA FLS

Time of treatment (hours)	Tau-Cl (μM)				Anti-Fas antibody (1 μg/ml)
	200	300	400	500	
24	0.6 ± 0.36	3.4 ± 2.2	11.6 ± 3.7$^{\#\#}$	11.2 ± 3.5$^{\#\#}$	18.1 ± 4.2$^{\#}$
72	0.9 ± 0.45	2.03 ± 0.85	8.5 ± 2.7	26.6 ± 3.5$^{\#\#\#}$	38.03 ± 7.3$^{\#\#\#}$

The cells were treated with indicated concentrations of either Tau-Cl or anti-Fas antibody. The activity of LDH was measured in the culture supernatants, as described in Materials and Methods. Data represent the percentage of cytotoxicity in relation to untreated control cells. Values are means ± SEM of 14-22 experiments. $^{\#}$ P = 0.05-0.01; $^{\#\#}$ P = 0.01-0.001, $^{\#\#\#}$ P = 0.001-0.0001 for control *vs.* either Tau-Cl- or anti-Fas-treated cells.

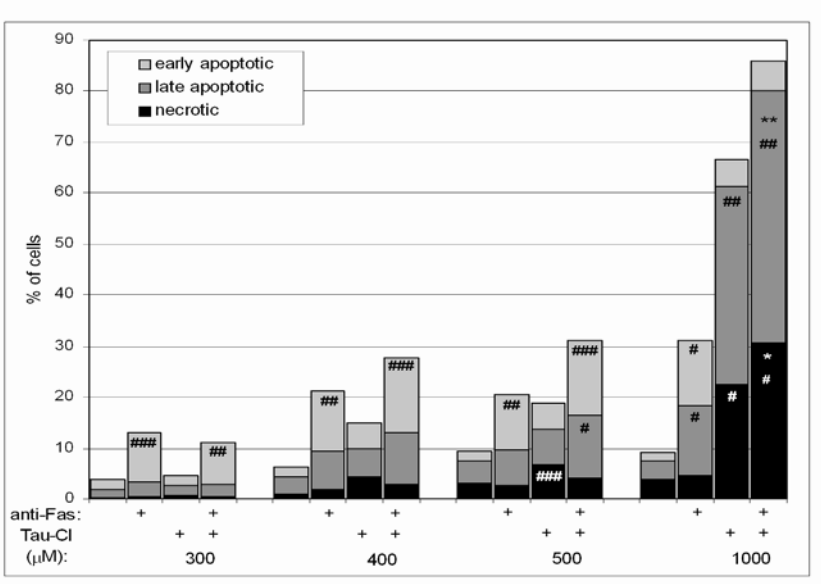

Figure 1. Effects of Tau-Cl and anti-Fas antibody on apoptosis and necrosis of RA FLS. The cells were treated for 24 hours with indicated concentrations of Tau-Cl and/or with anti-Fas antibody (1 μg/ml). Number of early apoptotic, late apoptotic and necrotic cells was calculated as described in Materials and Methods. Values are mean ± SEM of 3-8 experiments. $^{\#}$P = 0.05-0.01, $^{\#\#}$P = 0.01-0.001 and $^{\#\#\#}$P = 0.001-0.0001 for untreated control *vs.* either Tau-Cl- or anti-Fas-treated cells. *P = 0.05-0.01 and **P = 0.01-0.001 for Tau-Cl- *vs.* anti-Fas+Tau-Cl-treated cells.

resulting in more rapid (24 hours) death of 65 ± 9.9 % cells (Fig. 1). By contrast to Tau-Cl, treatment of the cells for 24 hours with anti-Fas antibody significantly raised the percentage of early apoptotic cells (Fig. 1), leading finally to death of 38.3 ± 7.3 % of cells after 72 hours (Table 1). In the co-treated cell cultures, only addition of 1 mM Tau-Cl significantly elevated the number of both late apoptotic and necrotic cells, as compared with anti-Fas treatment alone (Fig. 1). These results show that in RA FLS Tau-

Cl alone: (i) at the concentrations up to 400 μM has a negligible effect on RA FLS viability, while (ii) at higher concentrations it triggers either slow and mild (500 μM) or strong and rapid (1 mM) necrosis. Moreover, at the 1-mM concentration Tau-Cl shifts anti-Fas triggered apoptotic cell death toward the necrotic-like type.

3.1.2. Tau-Cl Influences Activity of Caspases

To further characterize Tau-Cl cytotoxicity, the effect of this compound on the caspases activities was evaluated. As shown in Figs. 2A and B, anti-Fas treatment triggered caspase 3 but not caspase 9 activity. By contrast, Tau-Cl alone had no effect on the activity of caspase 3, while at noncytotoxic concentrations slightly elevated activity of caspase 9, the enzyme attributed to mitochondrial pathway of apoptosis (Salvesen and Abrams, 2004). Interestingly, at the same concentration Tau-Cl inhibited anti-Fas-triggered activity of caspase 3 (Fig. 2C) and degradation of PARP (Fig. 2D). These results suggest that although Tau-Cl does not trigger typical apoptosis of RA FLS, it may influence cell death by modifying caspases activities.

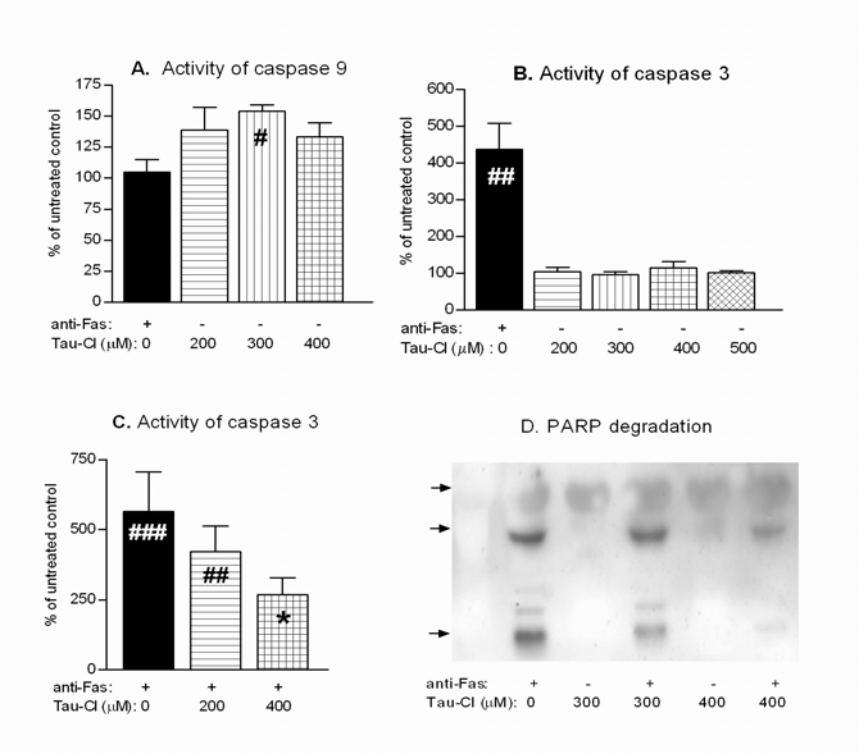

Figure 2. Activity of caspases and degradation of PARP protein in RA FLS. Cells were treated with the indicated concentrations of Tau-Cl and/or with anti-Fas antibody (1 μg/ml) for 4 (A) or 6 (B-D) hours. A-C: values are mean ± SEM of 3-10 experiments. $^{\#}$ P = 0.05-0.01, $^{\#\#}$ P = 0.01-0.001, and $^{\#\#\#}$ P = 0.001-0.0001 for untreated control *vs.* either Tau-Cl- or anti-Fas-treated cells. * P = 0.05-0.01 for Tau-Cl- versus anti-Fas+Tau-Cl-treated cells. D. Representative Western blot of 3 performed. The position of native (116 kDa) and degraded fragments (85 and 25 kDa) of PARP is marked with upper, middle, and lower arrows, respectively.

3.1.3. Tau-Cl Influences Subcellular Distribution of Bax

The effect of Tau-Cl on the subcellular distribution of the members of Bcl-2 family was further evaluated. In the untreated RA FLS, proapoptotic Bax protein was predominantly present in the cytosol, weakly expressed in the membrane and almost absent in the nuclear protein fractions. Treatment of the cells with noncytotoxic (400 μM) concentration of Tau-Cl resulted in a rapid (2-4 hours) accumulation of Bax protein in the nuclear fraction, with similar tendency for cytosol-to-membrane translocation of Bax protein (Figs. 3A and B). By contrast, the expression of antiapoptotic Bcl-2 protein in untreated cells was strong in the membrane, weaker in the nuclear (Fig. 3C) and negligible in the cytosolic (not shown) fractions, and Tau-Cl did not substantially affect the expression of this protein (Fig. 3C).

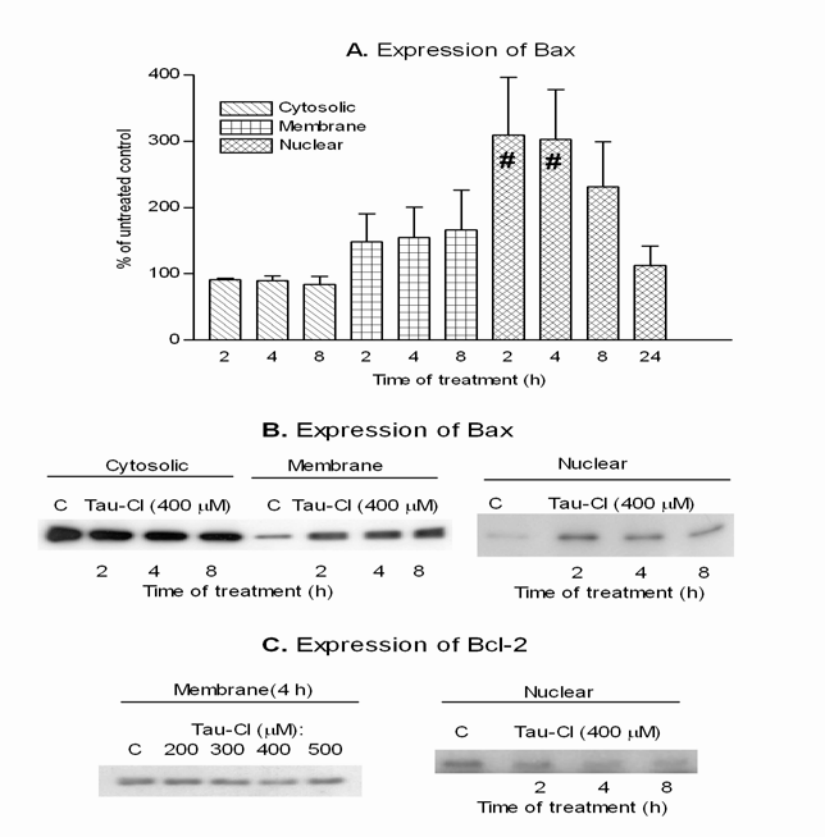

Figure 3. Effect of Tau-Cl on the subcellular expression of Bax and Bcl-2 proteins in RA FLS. The cells were treated with the indicated concentrations of Tau-Cl for 2-24 hours. The expression of Bax and Bcl-2 was estimated by Western blotting, as described in Materials and Methods. (A) Values are means ± SEM of 4-8 experiments. $^{\#}$ P = 0.05-0.01 for untreated control (C) *vs.* Tau-Cl-treated cells. Panels B-C are representative Western blots.

3.1.4. Tau-Cl Does Not Affect Cathepsin D Expression

Lysosomal proteases, cathepsins, translocate from the lysosomal lumen to the cytosol in response to a wide variety of death stimuli, including oxidative stress, and may trigger mitochondrial membrane permeabilization followed by either caspase-dependent or independent programmed cell death (Jäättelä, 2004). Therefore, the effect of Tau-Cl on the expression of cathepsin D was also evaluated. However, Tau-Cl at the 200-500 μM concentrations failed to modulate the expression of this enzyme in the cytosolic protein fraction (Fig. 4). Thus, implication of this enzyme in Tau-Cl triggered death of RA FLS is rather unlikely.

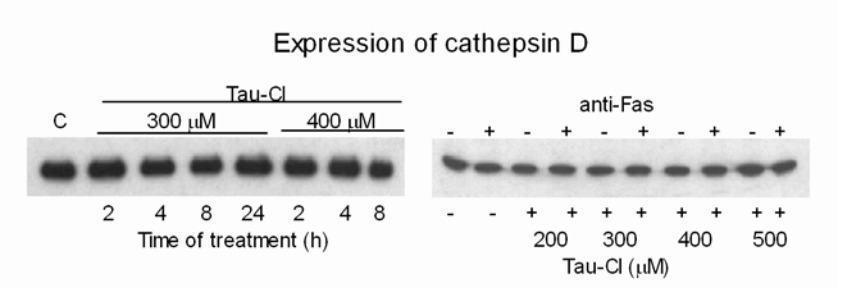

Figure 4. Tau-Cl fails to accumulate cathepsin D in the cytosol of RA FLS. The cells were treated for 2-24 hours with Tau-Cl alone (left panel) or for 24 hours with either Tau-Cl and/or anti-Fas (1 μg/ml) (right panel). The expression of cathepsin D was evaluated in the cytosolic fraction by Western blotting, as described in Materials and Methods. Representative Western blots of five blots done.

3.2. Jurkat Cells

3.2.1.Tau-Cl Triggers Apoptosis and Amplifies Anti-Fas-Triggered Cell Death

In Jurkat cells Tau-Cl induced cell death with characteristics of apoptosis: (i) at the concentration less than 300 μM raised the number of early apoptotic cells (Fig. 5), (ii) at the 300-μM concentration elevated the activity of caspase 9 (Fig. 6A), (iii) at ≥ 200 μM elevated the activity of caspase 3 (Fig. 6B) and the expression of processed, enzymatically active form of this enzyme (Fig. 6C), as well as degradation of PARP (Fig. 6D). All these events were dose-dependent and occurred between 4-8 hours of cell treatment. Interestingly, at the 300-400 μM concentrations Tau-Cl triggered apoptosis more efficiently than anti-Fas treatment (Figs. 5 and 6) and, when added together with anti-Fas antibody, even further enhanced apoptotic death of Jurkat cells. This effect was observed in every evaluated parameter of apoptosis (Figs. 5 and 6). After 18 hours both anti-Fas- and Tau-Cl-treated cells died by secondary necrosis because there was a significant increase of late apoptotic and necrotic cells, and Tau-Cl further raised the anti-Fas effect (Fig. 5).

3.2.2. Tau-Cl Fails to Influence Subcellular Distribution of Bax or Bcl-2

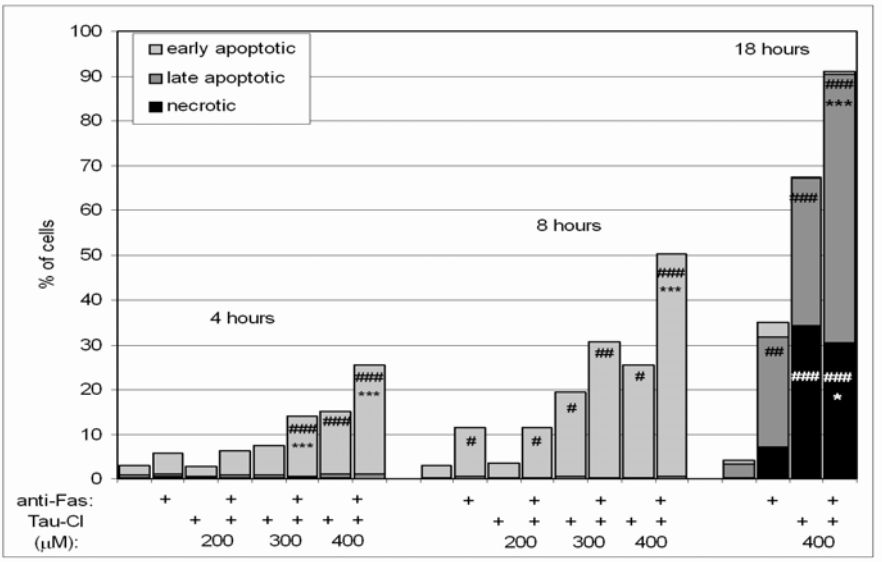

Figure 5. Tau-Cl triggers apoptosis and enhances anti-Fas-induced death of Jurkat cells. Cells were treated with indicated concentrations of Tau-Cl and/or with anti-Fas antibody (1 μg/ml) for 4-18 hours. Number of early apoptotic, late apoptotic, and necrotic cells was calculated as described in Materials and Methods. Values are mean ± SEM of 3-5 experiments. # P = 0.05-0.01, ## P = 0.01-0.001, and ### P = 0.001-0.0001 for untreated control versus either Tau-Cl- or anti-Fas-treated cells. * P = 0.05-0.01, and *** P = 0.001-0.0001 for Tau-Cl- versus anti-Fas+Tau-Cl-treated cells.

By contrast to some selectivity in subcellular distribution noted in RA FLS (Fig. 3B and C), in untreated Jurkat cells both Bcl-2 and Bax were strongly expressed in all tested protein fractions (Fig. 7A and B). Moreover, in these cells the level of Bax and Bcl-2 expression in the cytosolic, membrane, and nuclear fractions did not differ between control cells and the cells treated for 2-8 hours with 200-400 μM Tau-Cl. Because at this time Jurkat cells treated with ≥ 300 μM Tau-Cl died by apoptosis (Fig. 5), it seems that the distribution of Bax and Bcl-2 among particular subcellular compartments is not critical for Tau-Cl-triggered apoptosis in this cell line.

3.3.3. Tau-Cl Fails to Accumulate p53 Tumor Suppressor

As shown in Fig. 8, control Jurkat cells expressed high level of p53 protein in both cytosolic and nuclear fractions. This is consistent with the extendent half life and stabilization of p53 protein characteristic for tumor cells with p53 gene mutations (Cheng and Haas, 1990). Importantly, neither Tau-Cl nor anti-Fas antibody affected substantially expression of p53 protein in Jurkat cells. Thus, in Jurkat cells the ability of Tau-Cl to induce apoptosis and to enhance further anti-Fas-triggered programmed cell death is independent of p53 accumulation.

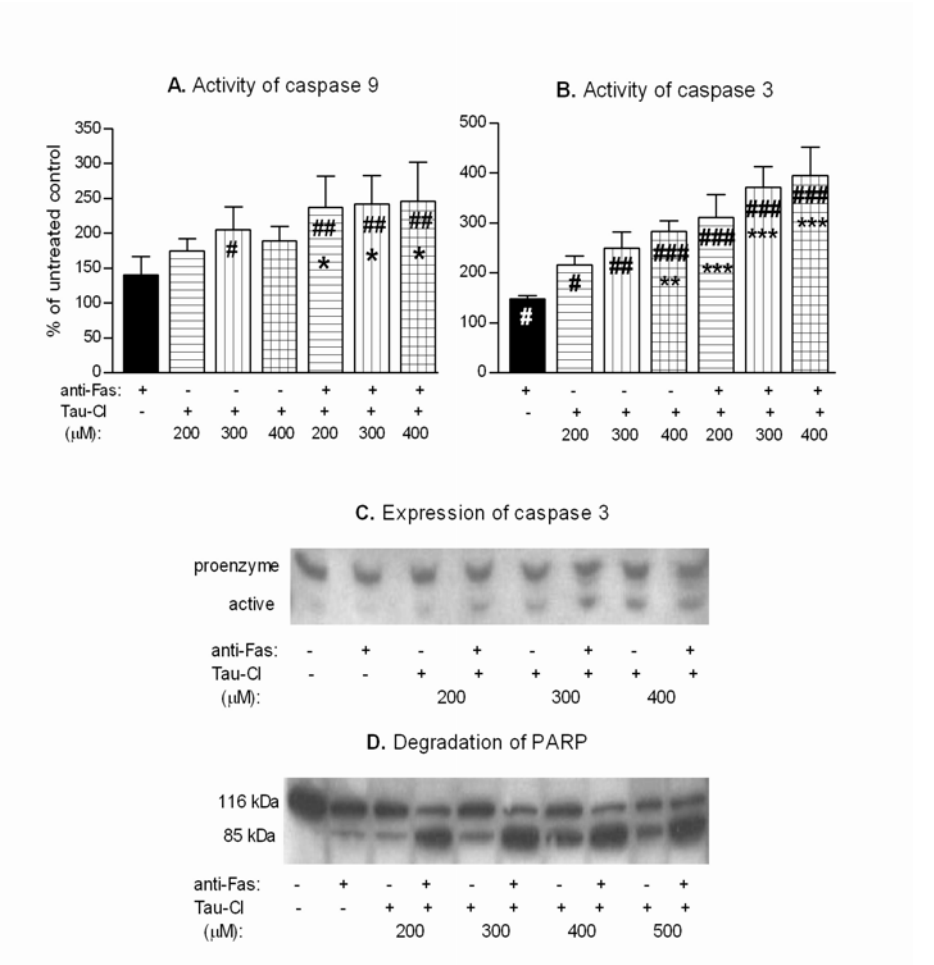

Figure 6. Activity of caspases and degradation of PARP protein in Jurkat cells. The cells were treated with indicated concentration of Tau-Cl and/or with anti-Fas antibody (1 μg/ml) for 4 (A) or 6 (B-D) hours. (A) and (B): the values are mean ± SEM of 4-5 experiments. $^{\#}$ P = 0.05-0.01, $^{\#\#}$ P = 0.01-0.001 and $^{\#\#\#}$ P = 0.001-0.0001 for untreated control *vs*. either Tau-Cl- or anti-Fas-treated cells. * P = 0.05-0.01, ** P = 0.01-0.001 and *** P = 0.001-0.0001 for Tau-Cl- *vs.* anti-Fas+Tau-Cl-treated cells. (C) Representative Western blot showing the expression of an inactive proenzyme and enzymatically active form of caspase 3. (D) Representative Western blot of 5 blots done. The position of native (116 kDa) and degraded fragment (85 kDa) of PARP is marked. See Materials and Methods for details.

4. DISCUSSION

Uncontrolled cell growth and death resistance is associated with many human pathologies. Cancer cells that have developed multiple death resistance strategies are typical examples (Jäättelä, 2004). In RA resistance of FLS to apoptosis and proliferation of these cells *in situ* results in hyperplasia of synovial membrane (Firestein, 1996).

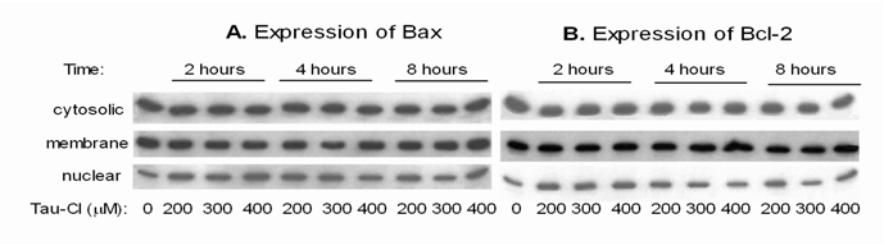

Figure 7. Tau-Cl fails to influence the subcellular distribution of Bax or Bcl-2 in Jurkat cells. The cells were treated with the indicated concentration of Tau-Cl for 2-8 hours. The expression of Bax and Bcl-2 was estimated in the cytosolic, membrane and nuclear protein fractions by Western blotting, as described in Materials and Methods. Representative Western blots of 3 blots done.

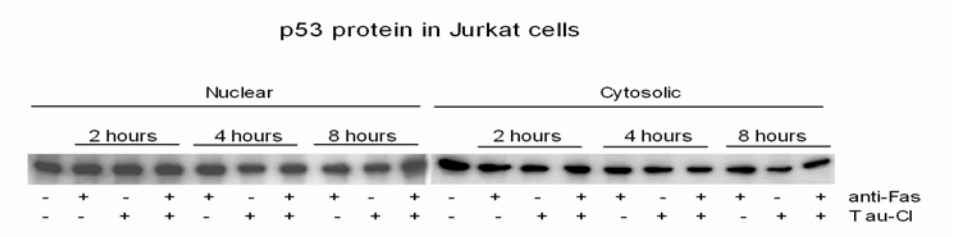

Figure 8. Tau-Cl fails to accumulate p53 protein in Jurkat cells. The cells were treated with either 300 μM Tau-Cl and/or with anti-Fas antibody (1 μg/ml) for 2-8 hours. The expression of p53 protein was evaluated in the nuclear and cytosolic fractions by Western blotting, as described in Materials and Methods. Representative Western blots of 3 blots done.

Cell death pathways vary, depending both on the type of lethal stimuli and the cellular context. Programmed cell death (PCD), an active cellular process driven by activation of aspartic proteases (caspases) or, as it has recently been pointed out, by other proteases, e g. lysosomal cathepsins (Jäättelä, 2004), is opposed to accidental necrosis in which the loss of control occurs without organized participation of proteases. According to the morphological criteria PCD is divided into classical apoptosis, apoptosis-like PCD and necrosis-like PCD. To take into account the death signaling pathways involved, PCD is also sorted according to the cellular compartment engaged in the process (e.g. membrane receptors, mitochondria, lysosomes, nuclei, endoplasmic reticulum). In the majority of PCD the mitochondrial membrane permeabilization (MMP) defines the point of no return. At this step apoptosis is tightly controlled by the Bcl-2 family members (Jäättelä, 2004). Proapoptotic members of this family (Bax, Bak) are pore-forming proteins that trigger the release of death-inducing mitochondrial proteins, while antiapoptotic members (Bcl-2, $Bclx_L$) oppose MMP presumably by heterodimerization with Bax-like proteins. Moreover, proapoptotic BH3-only proteins of this family (Bid, Bad, Noxa, etc.) promote apoptosis via direct binding and either activation of Bax-like proteins or inhibition of Bcl-2-like proteins functions. After the release from mitochondria, cytochrom C prompts the ATP-dependent assembly of apoptotic protease-activating factor 1 (Apaf-1) and caspase 9 into apoptosome, which forms the platform for caspase processing and activation. The initiator caspase 9, in turn, activates executioner caspase 3 (Salvesen and Abrams, 2004). Other mitochondrial proteins released activate

both caspase-dependent and -independent death pathways (Saelens *et al.,* 2004). The release of mitochondrial proteins has not been observed during necrosis and therefore it is thought to be characteristic for PCD (Saelens *et al.,* 2004). By contrast to this intrinsic mitochondrial pathway, PCD can also be initiated by ligation of death receptors (e.g. Fas/CD95), followed by the activation of initiator caspase 8 and executioner caspase 3. The receptor pathway is often enhanced by the caspase 8 mediated cleavage of BH3-only protein Bid that switches on the mitochondrial amplification loop (Krammer, 2000). Interestingly, in Fas-mediated apoptosis of RA FLS the mitochondrial pathway initiated by Bid cleavage has been shown to dominate and further amplified by secondary activated p53 tumor suppressor (Itoh *et al.,* 2004).

We have recently found that in RA FLS Tau-Cl at noncytotoxic concentrations triggers nuclear accumulation of p53 protein, followed by modulation of p53 transcriptional targets, including down-regulation of survivin (Kontny, *et al.,* 2005). Survivin is a protein that not only regulates cell division but also protects cells from apoptosis (Chiou *et al.,* 2003). These results suggested Tau-Cl to trigger apoptosis of RA FLS. However, the present work has not confirmed this assumption because we observed that at cytotoxic ($\geq$ 500 μM) concentrations Tau-Cl caused necrosis of these cells (Table 1, Fig. 1). Despite this, at lower noncytotoxic concentrations, Tau-Cl elevated the activity of caspase 9 (Fig. 2A) and triggered translocation of proapoptotic Bax protein from the cytosol to nuclear and membrane fractions (Figs. 3A and B). Both these events are associated with the mitochondrial pathway of apoptosis (Jäättelä, 2004; Salvesen and Abrams, 2004). Upon receipt of an apoptotic stimulus Bax undergoes a conformational change (Rathmell *et al.,* 2003), translocates to the membrane (Hsu *et al.,* 1997; Wolter *et al.,* 1997) and accumulates mainly in the nucleus (Aqeilan *et al.,* 2003). However, the role of Bax in the nucleus is still unknown (Aqeilan, *et al.,* 2003). Based on the present and previous results, we propose that at noncytotoxic concentrations Tau-Cl creates a proapoptotic state of RA FLS, manifested by accumulation of p53, translocation of Bax, elevation of caspase 9 activity and inhibition of survivin expression. Although in RA FLS these events are not sufficient to execute apoptosis (Fig. 1), all are characteristics for PCD. Importantly, we presently found that in the RA FLS the same noncytotoxic concentrations of Tau-Cl (Fig. 2C) significantly reduced the anti-Fas triggered activity of caspase 3. Accumulating evidence points out that inhibition of caspases sensitizes cells to necrosis and results in increased ROS (reactive oxigen species) formation either in mitochondria or lysosomes (Jäättelä, 2004). Whether Tau-Cl affects the functions of lysosomes needs further studies but our preliminary observation seems to question this, because we failed to observe any cytosolic accumulation of cathepsin D in the Tau-Cl-treated RA FLS (Fig. 4). Moreover, although survivin has been shown to protect cells from apoptosis by either directly or indirectly interfering with the function of caspases (Chiou, *et al.,* 2003), this mechanism of Tau-Cl inhibition of caspase 3 activity is also unlikely, because in the RA FLS Tau-Cl almost completely blocks the expression of survivin mRNA (Kontny, *et al.,* 2005). On the other hand, there are several ATP-dependent steps in apoptosis, including activation of caspases, and accumulating evidence shows that cells triggered to undergo apoptosis are forced to die by necrosis when the energy levels are rapidly compromised and drop below 25% of control (Englert and Shacter, 2002; Nicotera and Melino, 2004). One of the most cellular energy-consuming enzyme implicated in DNA damage-induced cell death is PARP (Cregan *et al.,* 2004) that causes poly ADP-ribosylation of many nuclear proteins. The activity of PARP increases 500-fold after binding to DNA strand breaks, leading to an excessive

consumption of cellular energy stores and necrosis, while the absence of this enzyme protects cells from a numbers of necrotic-like insults. In contrast, during apoptosis PARP is cleaved by caspases (mostly by caspase 3) into 85 and 25 kDa fragments to preserve sufficient energy for successful execution of caspase-mediated death. Noteworthy, in the RA FLS Tau-Cl did not cause PARP degradation but in a dose-dependent manner inhibited cleavage of this enzyme induced by anti-Fas treatment (Fig. 2D). Therefore, we conclude that in RA FLS Tau-Cl: (i) triggers several events typical for mitochondrial apoptosis pathway that do not result in execution of apoptosis, and (ii) switches anti-Fas-induced apoptosis into necrosis, probably due to depletion of the cellular ATP pool. Based on this, we propose to call this death the necrosis-like PCD.

By contrast, in Jurkat cells Tau-Cl-triggered apoptosis was accompanied by an increase in caspase 9 (Fig. 6A) as well as caspase 3 (Fig. 6B) activities and ending with an exposure of phosphatidylserine, visualized by annexin V binding (Fig. 5). Because subcellular localization of both Bax and Bcl-2 proteins was unaffected (Fig. 7), either the applied method was not sensitive enough to detect it or other Bcl-2 family members were more critical in regulating Tau-Cl-triggered apoptosis in these cells. Further studies are required to answer this question. Opposite to anti-Fas treatment, Tau-Cl alone significantly raised activity of caspase 9 (Fig. 6A), supporting previous observations of others that in lymphocyte cell lines Tau-Cl triggers the mitochondrial pathway of apoptosis (Emerson *et al.,* 2005). Moreover, we found that Tau-Cl significantly enhanced anti-Fas triggered apoptosis of Jurkat cells (Fig. 5) that was accompanied by a marked elevation of both caspase 9 (Fig. 6A) and caspase 3 (Figs. 6 B and C) activities. A similar effect has previously been reported in Jurkat cells pretreated with monochloramine (NH_2Cl) and stimulated with anti-Fas antibody (Ogino *et al.,* 2000). Thus, we conclude that in contrast to RA FLS, Tau-Cl facilitates anti-Fas-triggered apoptosis of Jurkat cells by inducing the mitochondrial amplification loop and very strong degradation of PARP protein (Fig. 6D), resulting probably in preservation of sufficient levels of ATP to execute apoptosis.

Another difference between RA FLS and Jurkat cells concerns the effect of Tau-Cl on p53 tumor suppressor expression. It is well known that in response to a variety of cellular stresses and insults, p53 protein is protected from proteosomal degradation and accumulates in the nucleus where acts mainly (but not exclusively) as a transcription factor regulating various genes required for the cell-cycle arrest, DNA repair, senescence, genomic stability and intrinsic pathway of apoptosis (Hofseth *et al.,* 2004; Slee *et al.,* 2004). In RA synovial tissue, dominant negative p53 mutation is rare and limited to a low number of cells (Sun and Cheung, 2002). Evidence from animal models of RA suggests that the up-regulation of functional p53 in RA synovial tissue represents an adaptive response with protective functions (e.g. cell-cycle arrest, apoptosis) (Pap *et al.,* 2001; Yao *et al.,* 2001; Yamanishi *et al.,* 2002). Consistently, we have recently reported that inhibition of RA FLS proliferation by Tau-Cl is preceded by nuclear accumulation of p53 with subsequent characteristic modulation of expression of the cell-cycle-associated genes (Kontny, *et al.,* 2005). These results indicate that in RA FLS Tau-Cl-accumulated p53 is transcriptionally active. By contrast, the present results show that in Jurkat cells Tau-Cl treatment failed to trigger nuclear accumulation of p53 protein (Fig. 8). Dominant negative mutations resulting in functional inactivation of p53 are characteristics for ~ 50% of human tumors, including Jurkat cells (Cheng and Haas, 1990), and are thought to contribute to tumor cell growth (Hofseth, *et al.,* 2004). Interestingly, the present results show that Tau-Cl triggers both p53-dependent and p53-independent cell death.

5. SUMMARY

We report that the effect of Tau-Cl on the cell fate strongly depends on the cellular context. In leukemic Jurkat cells Tau-Cl (>200 μM) triggers mitochondrial, p53-independent apoptosis and amplifies PCD induced by anti-Fas treatment. In contrast, Tau-Cl affects RA FLS in a dose-dependent manner. At the noncytotoxic (200-400 μM) concentrations it induces: (i) p53-dependent growth arrest (Kontny *et al.,* 2005), and (ii) Bax translocation and caspase 9 activity. Although the last events are characteristic for apoptotic state, there is not execution of RA FLS apoptosis, probably due to simultaneous inhibition of caspase 3 activity and prevention of PARP degradation. The last two events suggest an excessive ATP deprivation in Tau-Cl-treated RA FLS. At sufficiently high concentrations (≥ 500 μM) Tau-Cl causes therefore necrosis of these cells. Altogether our results suggest that Tau-Cl is able to eliminate the cells with both functional (RA FLS) and mutated (Jurkat) p53 tumor suppressor. This observation is clinically relevant because Tau-Cl is used in many animal inflammatory models and its sodium salt (used in this study) has been introduced to human therapy (Gottardi and Nagl, 2002; Teuchner *et al.,* 2005).

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EFFECTS OF DIETARY SALT AND FAT ON TAURINE EXCRETION IN HEALTHY AND DISEASED RATS

Mahmood S. Mozaffari, Rafik Abdelsayed, Champa Patel, and Stephen W. Schaffer*

1. ABSTRACT

Taurine modulates renal and cardiovascular function. Although the kidney regulates body taurine status, the impact of renal and cardiovascular risk factors, such as dietary intake of excess NaCl and saturated fat, on renal handling of taurine is less clear. One would predict that the kidney would modulate taurine excretion during dietary NaCl excess to insure adequate osmotic homeostasis. Similarly, fat feeding would be expected to affect taurine homeostasis, as taurine is involved in bile acid conjugation and therefore fat emulsification. To examine these aspects, male rats were divided into four groups: basal fat diet (control), high fat diet (FAT), basal fat and high salt diet (SALT) and a combination of a high fat and salt diet (FATSALT). While the control, FAT and SALT groups excreted similar amounts of taurine; the SALTFAT group excreted significantly more taurine than the other 3 groups. Although all of the dietary regimens increased renal tissue content of taurine, the increases were greatest in the two SALT groups. In a subsequent study, we examined the effect of excess dietary fat on taurine handling by the hypertensive (H) and hypertensive-glucose intolerant (HGI) rat. When fed a basal fat diet, the HGI group excreted more taurine than the H group, an effect likely related to increased endogenous taurine biosynthesis, alterations in renal function or a combination of the two effects. While excess fat intake increased urinary taurine excretion in the H group, it reduced taurine excretion in the HGI group. Nonetheless, kidney taurine content was similar in the 4 groups. Taken together, the data suggest that dietary constituents and preexisting systemic disorders are important modulators of renal handling of taurine.

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2. INTRODUCTION

Taurine is found in millimolar concentration in mammalian cells as opposed to micromolar concentration in the plasma. This gradient is maintained by an electrogenic plasma membrane transporter protein with a stoichiometry of 2-3: Na^+-1: Cl^--1: taurine. The plasma concentration of taurine is determined by dietary intake, hepatic biosynthesis and renal elimination. As a result, changes in dietary intake of taurine cause adaptive changes in proximal tubular reabsorption for taurine, thereby preventing marked changes in plasma taurine concentration (Handler and Kwon, 1993; Kine *et al.,* 1996; Matsell *et al.,* 1997; Lourenco and Camilo, 2002; Miyazaki *et al.,* 2004).

The high intracellular concentration of taurine contributes to the regulation of intracellular osmolality (Handler and Kwon 1993; Musch and Goldstein 2005). This aspect is of particular relevance to the kidney, in which the interstitial osmolality can become very hypertonic, an important feature of the countercurrent multiplier system that regulates the ability of the kidney to concentrate urine. As a result, in order to cope with the high osmolality of the interstitium, renal tubule cells accumulate non-perturbing organic osmolytes, such as taurine (Trachtman *et al.,* 1993; Amiry-Moghaddam *et al.,* 1994; Ma *et al.,* 1994; Nakanishi *et al.,* 1994; Pasantes-Morales *et al.,* 1998; Reymond *et al.,* 2000; Schaffer *et al.,* 2000).

In addition to its role in osmoregulation, taurine also affects kidney and cardiovascular function, including the modulation of renal excretory function, vascular reactivity and blood pressure (Abebe and Mozaffari, 2000, 2003a,b; Mozaffari and Schaffer, 2001, 2002; Militante and Lombardini, 2002; Mozaffari *et al.,* 2003). Yet it remains to be established whether altered body taurine homeostasis is associated with risk factors for renal and cardiovascular systems. Prominent among the environmental risk factors for the renal and cardiovascular systems are excess dietary intake of NaCl and saturated fat. Because of the consumption of foods rich in saturated fat, this dietary risk factor has assumed a central role in the worldwide epidemic of overweight and obesity (Freidman 2000, 2002). Further, such diets often contain elevated levels of NaCl, which is known to exacerbate blood pressure in susceptible individuals (Mozaffari and Wyss, 1999). It is likely that two events affect renal handling of taurine during excess dietary intake of NaCl and saturated fat. First, chronic exposure to dietary NaCl excess represents a state of osmotic stress. Because taurine is an important osmoregulator, alterations in body taurine homeostasis should accompany excess NaCl intake. Second, excess dietary intake of saturated fat should augment hepatic production of taurine, thereby providing substrate for the conjugation of bile acids that play a central role in fat emulsification and absorption of fatty acids (Lourenco and Camilo, 2002). To test this hypothesis, we examined the effect of dietary fat and NaCl excess on taurine handling by rats with normal renal function. In addition, we examined the impact of excess dietary fat on renal handling of taurine by NaCl-induced hypertensive and hypertensive-glucose intolerant rats; the combination of NaCl-induced hypertension and glucose intolerance impairs kidney function (Mozaffari *et al.,* 2003).

3. MATERIALS AND METHODS

3.1. Experiment 1: Effect of High Fat and High Salt Intake in Normotensive Rats

Male Wistar-Kyoto rats were obtained from Harlan Laboratories (Indianapolis, IN). Two days after arrival, the animals were randomly assigned to one of the following four groups (n=7 rats/group): (1) The control group consumed tap water and a basal fat diet (4.5% fat as calories, Harlan Teklad diet # 8604), (2) the high fat (FAT) group was provided with tap water and a high saturated fat diet (40% fat as calories, Harlan Teklad diet # 96132), (3) the SALT group was provided with the basal fat diet but tap water containing 1% NaCl, and (4) the FATSALT group was provided with the high fat diet and 1% NaCl drinking solution. The animals were maintained at constant humidity (60 ± 5%), temperature (24 ± 1^{o}C) and light cycle (0600-1800h) and had free access to food and drinking solution. Five weeks after initiation of the high fat diet, tail cuff blood pressure was measured on two consecutive days and the average of the two readings recorded for each rat (IITC Inc./Life Science Instruments, Woodland Hills, CA).

In order to determine urinary taurine excretion, animals were housed individually in metabolic cages between 5-6 weeks after initiation of dietary regimens. Following two days of acclimation, two consecutive urine samples were collected from each rat and food and fluid intake recorded. Six weeks after initiation of the dietary regimens, the animals were sacrificed and kidney weights measured. The renal tissue was then homogenized in ice-cold 2% perchloric acid (1:10, wt./vol.) and the supernatant used for measurement of tissue taurine content (Mozaffari and Schaffer, 2001). Urinary content of taurine was determined after precipitation of urinary proteins with 20% trichloroacetic acid (Mozaffari and Schaffer, 2001).

3.2. Experiment 2: Effect of High-Fat Diet on Hypertensive and Hypertensive-Glucose Intolerant Rats

Male and female Wistar-Kyoto breeder rats (Harlan Laboratories, Indianapolis, IN) were bred at the Medical College of Georgia animal facility. To produce impaired glucose tolerance, three-day-old male neonatal rats were injected intraperitoneally with 90 mg/kg of streptozotocin; control littermates received an injection of citrate buffer (0.1 M, pH: 4.5. The animals were weaned at 21 days of age and were maintained at constant humidity (60 ± 5%), temperature (24 ± 1^{o}C) and light cycle (0600-1800h). Unless otherwise specified, the animals had free access to food and water.

At four weeks of age, the streptozotocin-injected and control rats underwent a right nephrectomy during pentobarbital anesthesia (30-35 mg/kg; i.p.). Following a period of two weeks to allow for compensatory renal hypertrophy, impaired glucose tolerance was confirmed by the administration of a glucose tolerance test (Mozaffari and Schaffer 2002; Mozaffari *et al.,* 2003); and the test was repeated later at about 6 months of age. At 6 weeks of age, the uninephrectomized control and glucose intolerant animals were further subdivided and placed on a diet containing either high (8%) NaCl content (Harlan Teklad diet number 03003) or the combination of 8% NaCl and high fat 40% (Harlan Teklad diet number 02485). We have shown that dietary NaCl excess leads to the development of elevated blood pressure in the unilaterally nephrectomized rat (Mozaffari and Wyss, 1999; Mozaffari *et al.,* 2003). The protocol outlined above produced four experimental

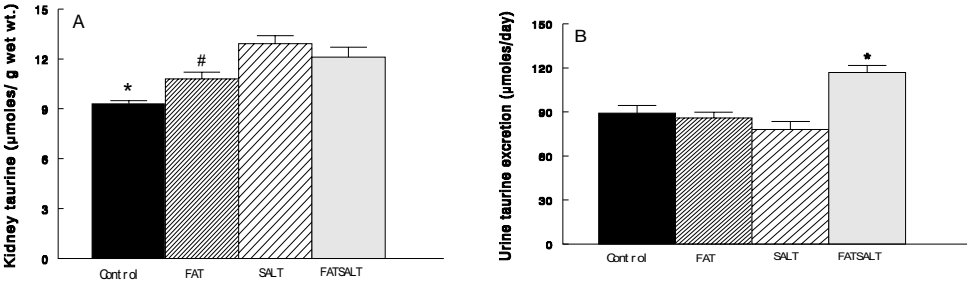

Figure 1: Increased dietary intake of saturated fat (FAT), high NaCl (SALT) or the combination of FAT and SALT (FATSALT) resulted in a significant increase in renal tissue taurine content (Panel A). By contrast, daily taurine excretion was significantly greater in the FATSALT group compared to the other three groups (Panel B). Data are means ± SEM of 7 rats/group. * $p<0.05$ compared to the other 3 groups; # $p<0.05$ compared to the other 3 groups.

groups (n=6-8 rats/ group) with the following designations: hypertensive control (H), fat-fed hypertensive control (fat-fed H), hypertensive-glucose intolerant (HGI) and fat-fed hypertensive-glucose intolerant (fat-fed HGI). At about six months of age, daily urine samples were collected for determination of urinary taurine excretion as described above. Body weight and tail cuff blood pressure were measured prior to sacrifice. Following sacrifice, the weight of the remaining kidney was recorded and the renal tissue processed for measurement of kidney taurine content as indicated above (Mozaffari and Schaffer, 2001).

All data were analyzed by one-way analysis of variance. Duncan's post hoc test was used for comparison of mean values (significance of criteria of $p<0.05$). Data are expressed as means ± SEM.

4. RESULTS

4.1. Experiment 1: Effect of High Fat and High Salt Intake on Normotensive Rats

Body weight was similar in the groups prior to placement on different dietary regimens. Body weight increased in all groups over time but the two fat-fed groups displayed greater ($p<0.05$) body weight [299 ± 4 g (FAT), 292 ± 9 g (FATSALT)] than those fed the basal fat diet [265 ± 5 g (control), 267 ± 6 g (SALT)]. The greater body weight of the two fat-fed groups (e.g., FAT and FATSALT) was associated with significantly lower food consumption compared to the control and SALT groups that were fed the basal fat (≈ 23%; $p<0.05$). Kidney weights were similar among the groups (range of 1.9-2.0 g). However, kidney size, expressed as kidney weight to body weight ratio, was lower in the two fat-fed groups [6.5 ± 0.1 (FAT) and 6.6. ± 0.2 (FATSALT) mg/g] compared to the other two groups [7.0 ± 0.1 (control) and 7.2 ± 0.1 (SALT) mg/g)], an effect due to greater body weight of the two fat-fed groups. Neither the dietary intake of excess NaCl nor the excess saturated fat diet significantly affected blood pressure (mean values for systolic arterial pressure in the range of 136-143 mmHg).

Renal tissue taurine content was greater in the three experimental groups than the control group, with the largest value seen in the SALT group (Fig. 1A). By contrast, urinary taurine excretion was similar in the control, FAT and SALT groups but significantly elevated in the FATSALT group (Fig. 1B).

4.2. Experiment 2: Effect of High-Fat Diet on Hypertensive and Hypertensive-Glucose Intolerant Rats

Systolic blood pressure was similar in the three experimental groups, with mean values in the range of 187-202 mmHg. Fasting blood glucose concentration was also similar in the groups. However, following intraperitoneal injection of a glucose load, the two streptozotocin-treated groups displayed a greater elevation in blood glucose concentration than their vehicle-treated control counterparts. Two hours after the glucose load, blood glucose concentration remained significantly higher (123-178%) in the two-streptozotocin-treated groups than the two vehicle-treated groups thereby indicating glucose intolerance. Body weight was similar in the groups prior to consumption of the high fat diet. While dietary fat excess did not affect body weight in the HGI group, the fat-fed H rats gained significantly more weight (≈ 65 g) over the course of the study than the other two groups. Kidney size, expressed as kidney to body weights, was greater (≈ 20-30%) in the fat-fed HGI than the other three groups which generally displayed similar kidney size.

Renal tissue content of taurine was similar in the experimental groups. By contrast, the HGI rats excreted more taurine than the H rats (Fig. 2A). Further, excess dietary fat intake significantly increased daily taurine excretion in the H group while decreasing it in the HGI group (Fig. 2B). As a result, the HGI group displayed greater taurinuria than the other three experimental groups (Fig. 2B).

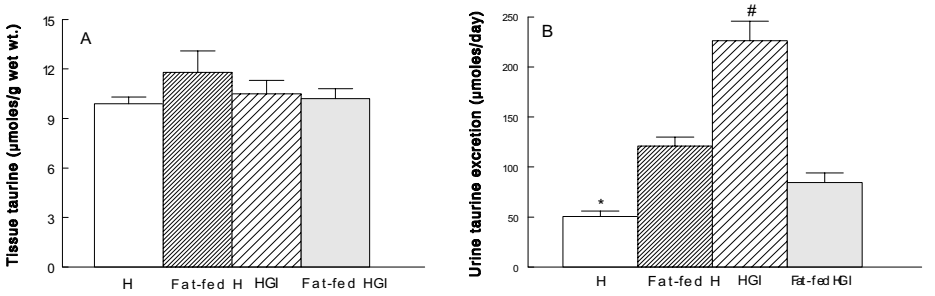

Figure 2: Increased dietary intake of FAT did not affect renal tissue taurine content of either the NaCl-induced hypertensive (H) or the NaCl-induced hypertensive-glucose intolerant (HGI) rats (Panel A). However, daily taurine excretion was significantly increased in the fat-fed H or HGI groups, with the effect being more marked in the latter group (Panel B). Data are means ± SEM of 6-8 rats/group. * $p<0.05$ H *vs.* HGI or H *vs.* fat-fed H. # $p<0.05$ compared to the other groups.

5. DISCUSSION

This study shows that increased renal tissue content and elimination of taurine accompanies excess dietary intake of NaCl and saturated fat in the normal rat. Similarly, the fat-fed NaCl-induced hypertensive rats excrete more taurine compared to their counterparts fed the basal fat diet. Interestingly, however, the HGI rats displayed the following distinguishing features: (a) greater taurine excretion in the basal fat-fed state compared to their hypertensive counterparts, likely related to increased endogenous taurine biosynthesis and/or altered renal function, and (b) reduced, rather than increased, taurine excretion when fed the high fat diet. Taken together, these observations suggest that renal taurine handling is modulated by dietary composition and pre-existing systemic disorders which affect kidney function and/or endogenous taurine biosynthesis.

Body taurine status is determined by exogenous intake of taurine and its precursor, cysteine, endogenous taurine biosynthesis, the activity of the taurine transporter and renal elimination (Reymond *et al.,* 2000; Mozaffari and Schaffer, 2001; Tappaz, 2004). Since dietary taurine content was not manipulated in this study, the observed changes in renal tissue taurine content and urinary taurine excretion in the experimental groups should be the net effect of the other three contributing factors.

Renal tissue taurine content was increased with excess intake of either SALT and/or FAT**,** indicating enhanced activity of the taurine transporter that regulates cellular uptake of taurine and/or elevated taurine synthesis by the kidney. Enhanced osmotic stress secondary to chronic SALT feeding is a plausible explanation for the increased accumulation of taurine by the NaCl-fed rats. This notion is consistent with a recent report**,** which showed overexpression of mRNA of the rat taurine transporter in the outer strip of outer medulla five hours after NaCl loading (Mitoun *et al.,* 2001). By contrast, the mRNA levels of enzymes for taurine biosynthesis, namely cysteine dioxygenase and cysteine sulfinate decarboxylase, were not affected by salt loading (Mitoun *et al.,* 2001). Nonetheless, the animals in our study consumed the high SALT diet for a protracted period of time and an effect of chronic SALT feeding on endogenous taurine production cannot be ruled out. Interestingly, dietary fat excess also caused a mild, but significant, increase in kidney taurine content. However, the effects of FAT and SALT were not additive, as kidney taurine content was similar in the three experimental groups. The reason for modulation of renal tissue taurine content by dietary FAT remains to be determined.

As opposed to dietary-induced renal accumulation of taurine, SALT feeding or FAT feeding alone did not significantly affect urinary taurine excretion. However, the combination of SALT and FAT treatments caused a significant increase in daily taurine excretion in either an otherwise normal rat (Fig. 1B) or in the H rats (Fig. 2B); concomitant SALT feeding and unilateral nephrectomy was used to induce systemic hypertension. It is possible that SALT and FAT interact at the level of the kidney to reduce tubular reabsorption of taurine and facilitate its excretion. Indeed, adaptive regulation of the taurine transporter in the proximal tubules is believed to play a pivotal role in regulation of body taurine status (Matsell *et al.,* 1997). However, the taurine transporter is Na^+ dependent, raising doubt about the reduction in reabsorption during salt excess. Rather, two lines of evidence suggest that increased endogenous (e.g., hepatic) taurine biosynthesis is primarily responsible for the significant taurinuria associated with the combination of excess FAT and SALT intake. First, dietary taurine content was not manipulated in this study. Second, increased renal taurine content rules out any

contribution of efflux/depletion of endogenous stores of taurine to the observed taurinuria. While tissues (e.g., kidney) possess the enzymatic machinery for taurine biosynthesis, hepatic production of taurine is the primary source of endogenous taurine (Lourenco and Camilo, 2002; Tappaz, 2004). The mechanism underlying the interaction between excess dietary intake of SALT and FAT and augmented endogenous production of taurine remains to be established. It is likely that the combined effects of osmotic stress secondary to high SALT intake and the greater need for bile acid conjugation because of excessive intake of FAT cause marked upregulation of endogenous (e.g., hepatic) taurine production; excess taurine is then eliminated by the kidney to maintain homeostasis.

One important aspect of the current study is the interaction of pre-existing systemic disorders (e.g., hypertension and impaired glucose tolerance) and renal handling of taurine. While hypertension alone or the combination of hypertension and impaired glucose tolerance did not significantly affect renal tissue taurine content, the HGI rats excreted significantly more taurine than their H counterparts. Plausible explanations for this finding include upregulation of endogenous taurine biosynthesis and/or impairment in the ability of the kidney to regulate taurine homeostasis. However, the latter is less likely because taurinuria in the HGI group was not accompanied with reduced endogenous taurine stores (e.g., kidney taurine content). Interestingly, the fat-fed NaCl-induced H group excreted more taurine than its H counterparts fed the basal fat diet. However, contrary to our expectation, the fat-fed HGI rats displayed a reduction, rather than an increase, in urinary taurine excretion. If one assumes that the endogenous taurine biosynthesis machinery is already maximally upregulated by the combination of NaCl-induced hypertension and impaired glucose tolerance, then feeding the HGI rat a high saturated fat diet would shunt more taurine towards bile acid conjugation and fat emulsification. Consequently, less taurine would be excreted by the kidney in order to maintain homeostasis.

In conclusion, dietary content of SALT and FAT, as well as glucose intolerance and hypertension, influence tissue content and renal elimination of taurine suggesting a dynamic interaction among dietary constituents and regulatory mechanisms for body taurine homeostasis (e.g., endogenous biosynthesis and renal elimination).

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CLINICAL SIGNIFICANCE OF PLASMA TAURINE

Part I. Plasma Taurine Reflects Sympathetic Tone

Tadaomi A. Miyamoto and Masumi R. Miyamoto*

1. INTRODUCTION

As we shall show in a later chapter in this volume, the baseline glycemia of non-recovering rabbits from spinal cord ischemia is higher than that of recovering animals but without differences in the osmolality. It suggests that the glycemia levels are constitutionally different even before ischemia. Because intravenously administered taurine has a major impact on glucose and functional results, it is hypothesized that plasma taurine might also be constitutionally different. To assess the role constitutional differences played in determining outcome, the rectoesophageal temperature difference, neurological functional status, blood pressure, baseline glycemia, and taurine levels in circulating plasma were linearly correlated.

2. MATERIAL AND METHODS

Spinal cord ischemia was induced in 12 placebo groups randomly assigned to ischemia times differing by 0.75 to one minute between groups covering the range of 12~2 minutes at the rigorously controlled esophageal temperature of 38.3^{o}C in 107 male Japanese white rabbits. We mechanically maintained eucapnia (expiratory [CO_2] 4.5~5.0 %) until anesthesia termination 30 minutes after re-perfusion (considered time 0), when switched to spontaneous air breathing. Blood pressure (n=107), glycemia (n=96), and taurine (n=6) responses to laparotomy and ischemia- reperfusion for 6 hours were analyzed. After laparotomy closure, the trachea was repaired while maintaining anesthesia (isoflurane 1%) with a cone-mask. Anesthesia was terminated 30 minutes after aorta reperfusion (=time 0) in all rabbits. The neurological function was assessed every 15 minutes after anesthesia termination with an objective neurological scoring (NS) from

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0=under anesthesia to 7=able to hop (Miyamoto *et al.,* 1998). The neurological outcome end-point was assessed 24 hours after the challenge as either fully (R+) regaining function or not. Less than full functional return was considered non-recovery, regardless of the neurological score, which included a group of animals which returned to NS7 once but deteriorated later, termed R+/-; and the animals that never regained function, termed R-/-, and combined as R(-).

2.1. Statistical Analyses

We used the commercially available software PRISM version 3.0 (Graph Pad Software Inc, San Diego, CA) for analysis of all data. The values were expressed as mean ± SD, but mean ± SEM bars are used in all figures for clarity purposes. Independent variables were compared using Mann-Whitney test, and/or one-way ANOVA with Tukey's multiple comparison test for comparing more than 3 subgroups, or two-way ANOVA followed by Bonferroni post-test when comparing recovery courses. Correlation was analyzed by linear regression: $p<0.05$ was considered significant.

3. RESULTS

Table 1. Baseline data; recovered *vs.* non-recovered

No of experiments % of total (N=107)	49 0.46	R(+)		58 0.54	R(-)
	Mean±SD	2-way ANOVA P value	Bonferroni's, or Tukey's test (post-ANOVA) P	Mean±SD	
Rectal Temp.	39.02 ± 0.47		ns	38.93 ± 0.40	
Esoph. Temp.	38.45 ± 0.45		ns	38.61 ± 0.41	
R-ET	**0.52 ± 0.38**		**<0.01**	**0.30 ± 0.34**	
Mean BP	90.68 ± 20.35		ns	90.10 ± 19.64	
Glucose	**13.53 ± 3.64**	**0.0257**	**<0.05**	**15.54 ± 3.98**	
Taurine	**95.33 ± 13.00 (n=4)**	**0.0008**	**<0.01**	**209.9 ± 56.00 (n=2)**	

Abbreviations: BP=blood pressure; N=number; R-ET=rectal-esophageal temperature difference; R(+)=recovered; R(-)= non-recovered.

3.1. Recovered and Non-recovered Animals Are Different Before the Experiment

3.1.1. Recto-Esophageal Temperature Difference

The rectal temperature was significantly higher than the esophageal in all animals but there were no differences between the two groups (Table 1). When the recto-esophageal temperature differences (R-ET) for each animal were compared, the gradient in the R(+) animals was significantly greater than that in the R(-) animals (Fig. 1A).

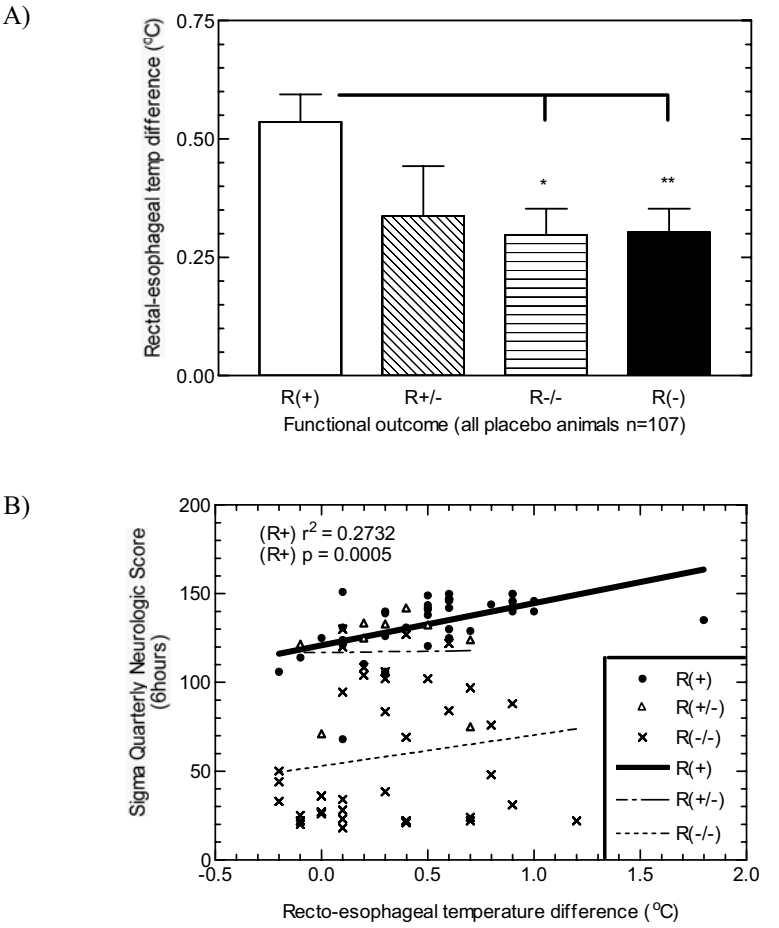


Figure 1. (A) The recto-esophageal temperature gradient in the R+ animals is significantly greater than that in the R- animals. (B) The speed of recovery as assessed by the sigma of quarterly neurological scores correlates positively with the temperature difference in the recovering animals. Mean±SEM bars are used throughout all figures.

3.1.2. Baseline Glycemia in Non-recovered Animals Is Higher than in Recovered Animals. Blood Pressure and Glycemia Response Depend on Neurological Status

Neurological end-point evaluation was available in all 49 (46%) recovered and 58 (54%) not-recovered animals (Fig. 2A). The functional recovery status determines the blood pressure and glycemia response after anesthesia termination. The blood pressures before, during aortic clamping and for the first 30 minutes post-reperfusion were similar. However, in the R(-) animals higher pressures started to occur 45 minutes post-anesthesia termination. At 90 minutes the blood pressure curve was higher, almost reaching significance (two-way ANOVA p=0.0591, Fig. 2A). While the R-ET gradient correlated positively with the sigma neurological score only in the R(+) animals (Fig. 1B), the

sigma neurological score correlated negatively with the baseline glycemia only the in R+/- but not in the R(+) or R-/- animals (Fig. 2C).

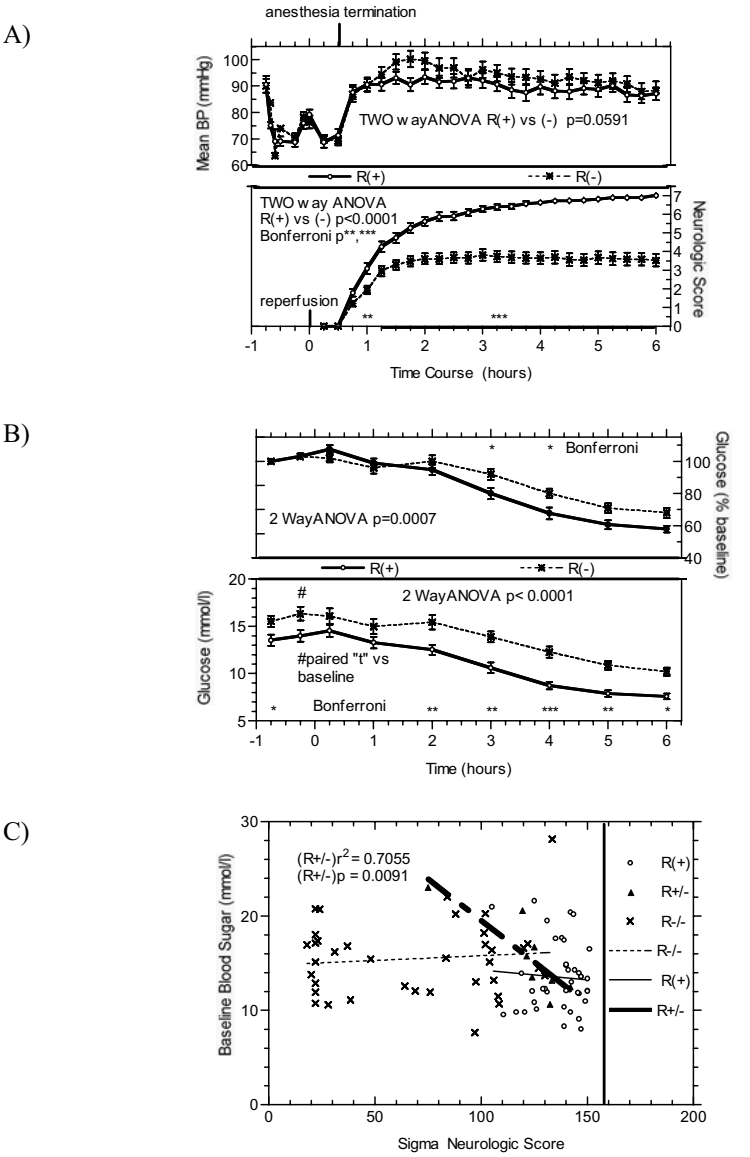
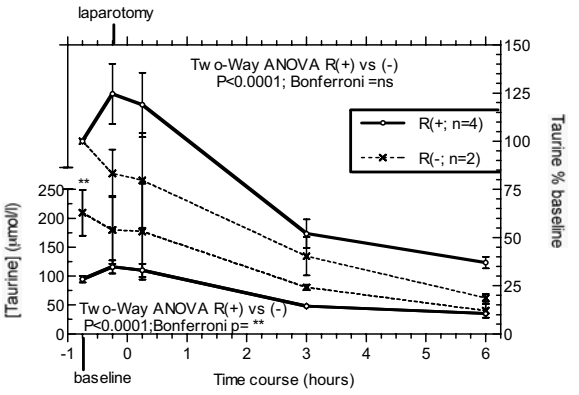


Figure 2. The neurological functional status determines the mean blood pressure (BP) and glucose responses after post-anesthesia termination. (A) The BP in the non-recovered R(-) animals is distinctly higher than in the R(+) animals, two-way ANOVA p is almost significant. (B) Significantly different glucose responses were observed at 150 and 210 minutes (=3.4 h post-reperfusion) after post-anesthesia termination. The glucose concentration in R(-) > R(+) (*) at baseline (Bsln). (C) Sigma neurological score correlates negatively with the baseline glucose levels in the R+/- animals but not in the R+ or R-/- animals.

3.2. Baseline Taurine Concentration in Non-Recovered Animals Is Higher Than That in Recovered Animals. Taurine and Glycemia Levels Correlate

A)



B)

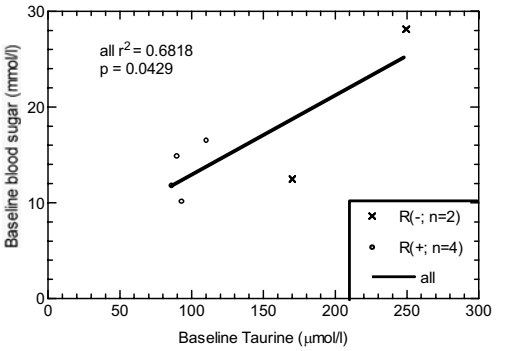


Figure 3. (A) Baseline taurine concentration in the R(-) animals is higher (**) than that in the R(+) animals and the time courses are entirely different, even before the ischemic event. (B) Baseline taurine and blood sugar concentrations correlate.

3.3. Taurine Responses Occur at Laparotomy Before the Ischemic Event. Glycemia Changes After Anesthesia Recovery

While taurine increases in the recovered and decreases in the non-recovered animals in response to the surgical stress of laparotomy, glycemia increases in both groups to the same extent. However, the significant difference of the non-recovered animals' baseline glycemia becomes even greater after anesthesia termination (Fig. 4).

4. DISCUSSION

Lactic acidosis mediates hyperglycemic damage (Siesjö *et al*., 1996). Detrimental effects of hyperglycemia in CNS ischemia are a threshold function of the glucose levels: 15>12>10 mmol/kg (Li *et al.,* 1994, 1995, 1997, 1999, 2000, 2001). Our studies

confirmed those observations, but the baseline glucose levels could not be used to predict the neurological outcome, since this varies with the duration of ischemia and temperature, as we show in the later chapter in this volume. Whatever determines glycemia at rest, seems to determine the eventual neurological outcome as well.

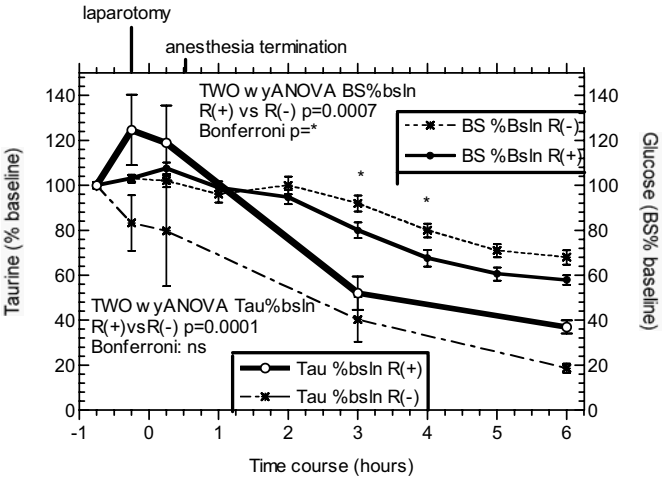


Figure 4. The response of taurine to laparotomy before the ischemic event in R(+) animals is in an opposite direction to that in R(-) animals, while the glycemia responses to ischemia/reperfusion become significantly different after anesthesia effects wear out, that is, being dependent on the neurological function.

4.1. What Recto-esophageal Temperature Difference Represents. The Neurological Outcome Might Be Predetermined Before Ischemia Lasting 2~5 Minutes

The significantly smaller baseline recto-esophageal temperature difference in the R(-) animals than in the R(+) animals is noteworthy. Though the difference is only 0.2~0.25°C, it indicates a warmer central mixed blood temperature. It implies a decreased cooled venous return (peripheral or subcutaneous blood) relative to the total (peripheral + central or core organs with large blood flow) venous blood volume due to the greater peripheral vasoconstriction (greater sympathetic tone) than in the R(+) animals causing a higher temperature in left atrial/aortic blood and consequently in the esophagus because of anatomical proximity. The spinal cord is normally warmer (0.2~0.3°C; Ohno *et al.,* 1999), but physiologically cooled by arterial blood. A higher aortic blood temperature decreases the central nervous system (CNS) cooling efficiency in spite of similar blood pressures/flows, resulting in a higher CNS temperature that decreases the ischemic tolerance. In other words, a higher sympathetic tone predisposes to CNS injury. Although in the present experimental conditions the esophageal temperatures at the time of ischemia induction were controlled to be similar, the animals with a greater recto-esophageal temperature gradient at baseline (less sympathetic tone, i.e., greater cooled peripheral venous return) might be more efficient in lowering the temperatures in blood and consequently in the CNS after reperfusion. Early post-ischemic hypothermia is known to be protective. Even a small post-reperfusion temperature drop might be as

important as the intra-ischemic ones, for which 0.5°C difference resulted in the opposite outcomes after 60-min ischemia (Ohno *et al*., 1999); thus for shorter ischemia times, smaller differences might be enough (see the later chapter).

Baseline blood glucose in the R(-) animals is higher than in the R(+) animals. Laparotomy increases similarly the glucose concentration in both groups, but the differences between the R(-) and R(+) animals get worse as anesthesia wears out, that is, when being functionally impaired becomes a major stress component in the sympathetic tone (Fig. 2B). The baseline glucose levels correlating negatively with the sigma neurological score in the R+/- animals but not with the baseline rectal-esophageal temperature difference indicate that glucose levels play the major role in determining the neurological deterioration in this particular subset. It suggests that the detrimental effects of hyperglycemia dominate the naturally recruited protective mechanisms when duration of ischemia increases from 2 to 5 minutes (Benveniste *et al.,* 1984; Huxtable, 1989; Blondeau *et al.,* 2002). On the other hand, the baseline rectal-esophageal temperature difference correlating positively only with the sigma neurological score in the R+ animals suggests that if destined to recover, its speed is determined by the prevailing temperature distribution before and after the ischemic event, that is, the sympathetic tone.

In spite of the small number of animals having the concentration of taurine determined, the baseline taurine level in the R(+) animals was not only different from that in the R(-) animals but also correlated positively with the glucose concentrations (Fig. 3B). Because of the small numbers only a trend was observed when the R-E temperature difference was correlated with the taurine concentration. Nevertheless, it suggests that the functional outcome after ischemia of 2~5 minutes might be predetermined by the constitutional factors controlling the ratio of peripheral *vs.* central venous return, i.e., the peripheral vascular resistance whose major determinant is the sympathetic tone (stress) and reflected as circulating taurine levels. Furthermore, the response of taurine to laparotomy (a new surgical stress) before the ischemic event in the R(+) animals being in an opposite direction to that in the R(-) animals (Fig. 4) suggests that the taurine concentration may potentially be used to predict the eventual neurological outcome following an ischemic insult.

4.2. Limitations of the study

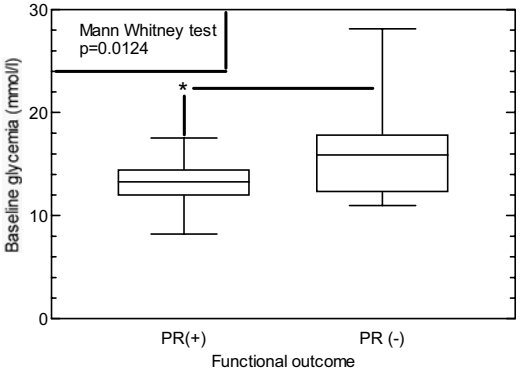
The number of animals with baseline taurine determinations is only 6. For definitive conclusions, a greater number of experiments is mandatory.

Part II. Spontaneously High Plasma Taurine Concentration Presages Bad Outcome If Challenged with Ischemia

1. INTRODUCTION

Our findings corroborated the previously reported detrimental role of hyperglycemia on brain ischemic tolerance (Siesjö *et al.,* 1996; Li *et al.,* 1994, 1995, 1997, 1999a,b, 2000, 2001; Alberti *et al*., 2000;). Taurine has been attributed the function as Ca^{2+} homeostatic agent in the brain (Lehmann *et al.,* 1984). Our data suggests an important role in glucose homeostasis as well. The baseline plasma taurine level was found to correlate with the plasma glucose concentration and neurological function after an ischemic challenge in a small number of experiments. We evaluated whether the same correlation could be found in a larger group of animals having the benefits of protective strategies.

A)



B)

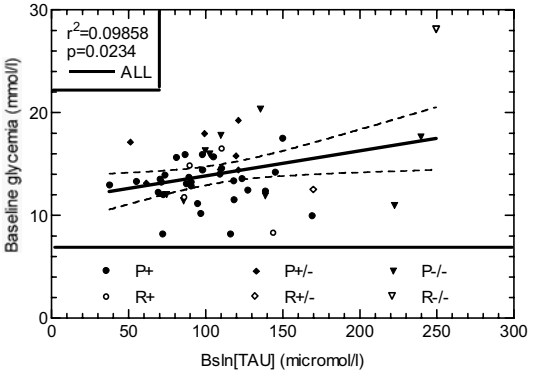


Figure 5. (A) Baseline glucose concentrations in R(-) are greater than in R(+), the entire mixed group (n=51), and (B) correlate positively with taurine.

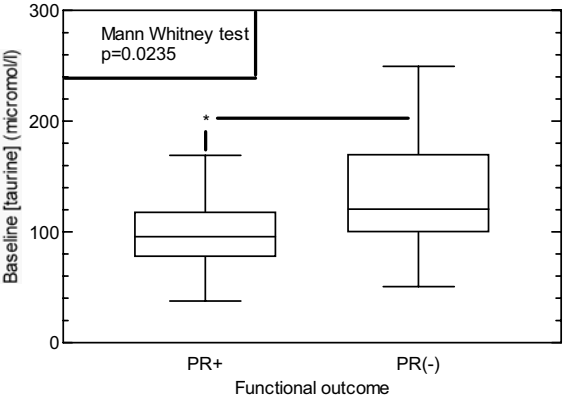
2. MATERIAL AND METHODS

Six placebo animals that had taurine measured at baseline were mixed with the study animals (n=45/66) given taurine subsequently to assess its protective mechanisms. The baseline taurine and glucose concentrations in addition to the rectal-esophageal temperature differences were correlated.

3. RESULTS

The R-E temperature differences of all functional subsets were similar but both baseline taurine and glucose concentrations in the PR(-) animals were significantly greater than in the PR(+) animals (Fig. 5A, Fig. 6A). The baseline taurine level correlated positively with the glucose level in all animals (Fig. 5B) but negatively only with the R-E temperature in the PR(+) subset of animals (Fig. 6B).

A)



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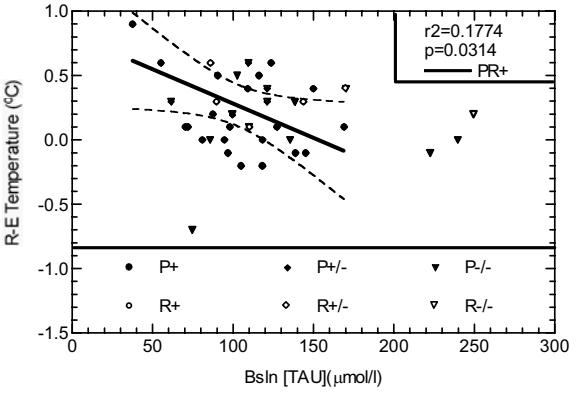

Figure 6. (A) Baseline taurine concentrations in PR(-) are greater than in PR(+), the entire mixed group (n=51), and (B) correlate negatively with the R-E temperature difference only in the PR(+) animals.

4. COMMENTS

This study involving a greater number of animals corroborated the observations outlined previously. The R-E temperature difference was similar in all functional subsets, which is apparently non-congruent with the conclusions in part I. However, it could be explained on the basis of baseline data being arranged according to the functional outcome. The protected taurine group includes a number of animals with a decreased R-E temperature difference, which would have failed to recover without taurine. The R-E temperature gradient correlated negatively with the baseline taurine concentration in protected/recovered animals, that is, the role played by the sympathetic tone was still manifested as a faster recovery in animals with larger temperature differences or lower sympathetic tone. The fact that taurine and glucose concentrations correlated positively in this larger group supports the contention that the baseline taurine level in placebo animals rather than in the combined group should be used as a prognostic indicator. The baseline taurine concentration in the combined protected/recovered group is higher than that in the recovered/placebo groups, since it includes animals with a high taurine concentration, which would have failed to recover without taurine. In this context it is important to notice that the taurine levels are decreased in pregnant diabetic rodents (Aerts and Van Assche, 2001), which may be detrimental because it might indicate depletion either from inadequate intake/synthesis and/or excessive losses/utilization and inability to cope with additional stress.

Although the observations were made on a spinal cord ischemia model, we assume it could be extended to the brain. Initial hyperglycemia has been used as an indicator of severity of the ictus in poor-grade patients with spontaneous subarachnoidal hemorrhage (Alberti *et al.,* 2000). Hyperglycemia is the biochemical manifestation of stress or the sympathetic tone but may be less sensitive than the taurine concentration, which might be of greater prognostic value than glycemia. Taurine may thus play a role as important as insulin in glucose homeostasis, not only in CNS Ca^{2+} homeostasis (Lehmann *et al.,* 1984).

4.1. Limitations

Ideally taurine should have been determined in a larger group of placebo animals to be conclusive. Nevertheless, the findings supplement and support the previous contentions.

5. ACKNOWLEDGMENTS

We thank Dr Hiroshi Takeshita, the former hospital director, for allocating funds to the Research Department, Dr Toshihiko Ban, the succeeding director, and the clinical laboratory staff for their continued encouragement and support given to me during my tenure, and the SRL (Tokyo) staff for determining amino acids.

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EFFECTS OF TAURINE ON mRNA LEVELS OF NUCLEAR RECEPTORS AND FACTORS INVOLVED IN CHOLESTEROL AND BILE ACID HOMEOSTASIS IN MICE

Nien Vinh Lam, Wen Chen, Kazuhito Suruga, Naomichi Nishimura, Toshinao Goda, Hiroaki Oda, and Hidehiko Yokogoshi*

1. INTRODUCTION

Cholesterol conversion to bile acids plays a vital role for elimination of cholesterol, which is one of the main factors regulating cholesterol homeostasis in the body (Sjovall, 2004). This process occurs via the "classic" (neutral) or the "alternative" (acidic) bile acid biosynthesis pathways (Javitt, 1994). The first and rate-limiting reaction of the classic pathway is the 7α-hydroxylation of cholesterol, which is catalyzed by cholesterol 7α-hydroxylase, a product of a liver-specific microsomal cytochrome P450 gene (CYP7A1) (Myant and Mitropoulos, 1977; Jelinek *et al.,* 1990; Nelson *et al.,* 1996). There is evidence suggesting that the transcriptional level of cholesterol 7α-hydroxylase is regulated by a wide range of diverse stimuli including bile acids, steroid hormones, thyroid hormones, cytokines, insulin, retinoids and cholesterol (Li *et al.,* 1990; Russell and Setchell, 1992; Chiang, 1998). Furthermore, many nuclear receptors have been reported to regulate the transcription of CYP7A1 gene. Two regions for transcription factor binding (bile acid response elements, BARE-I and BARE-II) have been identified in the CYP7A1 promoter (Stroup *et al.,* 1997). BARE-I in mouse binds LXRα (liver X receptor α), which induces CYP7A1 gene transcription with oxysterols as its ligands (Lehmann *et al.,* 1997). In human, LXRα binding site is not present in the CYP7A1 gene (Agellon *et al.,* 2002). BARE-II contains binding regions for HNF-4α (human nuclear factor 4α) and LRH (liver related homologue), which were reported to be essential for basal level expression of CYP7A1 (Crestani *et al.,* 1998; Nitta *et al.,* 1999). FXR (farnesoid X receptor) is a bile acid receptor and represses CYP7A1 transcription by

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promoting the transcription of the atypical nuclear receptor small heterodimer partner (SHP). The ATP-binding cassette transporters ABC-G5 and ABC-G8 exist in the hepatocytes and enterocytes and are induced by LXRα to enhance cholesterol efflux into biliary ducts and intestinal lumen (Yu *et al.,* 2002). BSEP (bile salt exporter pump) and I-BABP (ileal bile acid binding protein) are induced by FXR to excrete bile acids into bile and enhance ileal bile salt re-absorption, respectively (Grober *et al.,* 1999). I-BAT (ileal bile acid transporter) is another factor that plays pivotal role in active intestinal bile acid absorption (Hagenbuch and Dawson, 2004).

Taurine, 2-aminoethanesulfonic acid, which is present freely at high concentrations in mammalian plasma and cells, has been reported to play an important role in several essential biological and physiological functions such as brain and retinal development, calcium modulation, membrane stabilization, reproduction, immunity, antioxidation, detoxification and osmoregulation (Kuriyama, 1980; Thurston *et al.,* 1980; Pasantes-Morales *et al.,* 1985; Wight *et al.,* 1986; Huxtable, 1992; Sturman, 1993). In recent years, the roles of taurine in cholesterol metabolism have appealed much attention. We reported the improving effects of taurine on cholesterol profile in rats and mice (Mochizuki *et al.,* 1998; Yokogoshi *et al.,* 1999; Nishimura *et al.,* 2003; Chen *et al.,* 2004). Taurine also demonstrated the cholesterol-lowering effect in other species, such as hamsters, guinea pigs, rabbits and human (Herrmann, 1959; Cantafora, 1986; Murakami *et al.,* 2002; Zhang *et al.,* 2004). We also reported that the hypocholesterolemic effects of taurine were mainly due to the enhancement of cholesterol degeneration and the excretion of bile acids via its increasing effect on CYP7A1 mRNA activity and mRNA level in rats (Yokogoshi *et al.,* 1999).

In this study, in order to reveal the molecular mechanism of cholesterol-lowering effect of taurine, we investigate the effect of taurine on mRNA levels of some nuclear receptors and factors involved in cholesterol and bile acid homeostasis.

2. MATERIALS AND METHODS

2.1. Animals and Diets

Male C57BL/6 mice aged 10 weeks were obtained from Japan SLC (Hamamatsu, Japan). The animals were maintained at 23 ± 1°C with 55±10% humidity and a 12-h light (07:00-19:00) and dark cycle. The mice were accustomized to experimental conditions by being fed with control diet (20% casein) in 3 days. On the 4th day, they were divided into 6 groups each with 4 mice: control group (N), high cholesterol diet group (C), high cholesterol and sodium cholate group (CB), and their 1% taurine-supplemented groups (NT, CT, and CBT, respectively) based on their serum cholesterol levels and body weights. Diet composition in each group is shown in Table 1. The mice had free access to water and the respective diet for one week. Feces in the last 48 hours were collected, lyophilized and stored at –20°C until assay. After the cholesterol-lowering effect of taurine was confirmed by determining serum cholesterol, the mice were anesthetized with diethyl ether and decapitated at 23:00. Blood was collected and liver, jejunum and ileum were removed immediately for quantification of mRNA levels of nuclear receptors and other key factors.

The experimental procedures used in this study met the guidelines of the Animal Care and Use Committee of the University of Shizuoka.

Table 1. Diet composition (g/kg)

Groups	N	NT	C	CT	CB	CBT
Casein	200	200	200	200	200	200
Cellulose	50	50	50	50	50	50
Corn oil	50	50	50	50	50	50
Choline cloride	1.5	1.5	1.5	1.5	1.5	1.5
Vitamin mix AIN-93	10	10	10	10	10	10
Mineral mix AIN-93G	35	35	35	35	35	35
Sucrose	217.8	214.5	214.5	211.1	213.7	210.3
Corn starch	435.7	429	429	422.4	427.3	420.7
Cholesterol	---	---	10	10	10	10
Sodium cholate	---	---	---	---	2.5	2.5
Taurine	---	10	---	10	---	10

2.2. Measurement of Fecal Bile Acids and Neutral Sterol

Lyophilized feces were ground and steroids were extracted by ethanol and petroleum ether as described by Sheltawy and Losowsky (1975). The extract was dried under N_2 and then resuspended in methanol. Total bile acids were determined by an enzymatic method. Fecal neutral sterol was detected according to Moundras *et al*. (1997).

2.3. Determination of CYP7A1 Activity

Mouse liver microsomes were isolated and CYP7A1 activity was determined according to the description of Ogishima and Okuda (1986). 7α-Hydroxycholesterol was used as the standard.

2.4. Total RNA Preparation and Northern Blot Analysis

Total RNA was extracted from the mouse liver and jejunum using TRIzol (Invitrogen, USA) according to the manufacturer's instructions and stored at -80°C until assay. For Northern gel analysis, equal aliquots of total RNA (35 μg) were subjected to electrophoresis on 1% agarose/formamide gels and blotted onto Hybond-XL membranes (Amersham Pharmacia Biotech, UK). Blotted membranes were hybridized with a ^{32}P-labeled cDNA probe at 42^{o}C overnight. After washing, the membranes were autoradiographed (BAS cassette, Fujix) and the resulting bands were analyzed by exposing to Storm 820 Molecular Dynamics (Amersham Pharmacia Biotech). The mRNA expression levels were corrected with regard to the corresponding GAPDH mRNA obtained from the same membrane.

2.5. Statistical Analysis

The means and SD of 3 mice per group were reported for mRNA quantification, and of 4 mice per group were reported for other indexes. Statistical analysis was performed by one-way ANOVA followed by Duncan's multiple range test. *P*-values of less than 0.05 were considered to be statistically significant.

3. RESULTS

The fecal bile acid level was markedly increased in C group compared to that of the N group and apparently further increased by the sodium cholate load in the CB group (Table 2). The fecal bile acid excretion was further increased by taurine supplementation in both CT and CBT groups compared to C and CB groups, respectively. Neutral sterol in feces was also significantly increased by loading of diet cholesterol, but taurine did not show clearly effect on this index. These data suggest that cholesterol degradation is enhanced by cholesterol loading in the diet and is further promoted by taurine supplementation.

Table 2. Fecal bile acids and neutral sterol

Groups	N	NT	C	CT	CB	CBT
Feces weight (g/2 d)	0.72 ± 0.03	0.75 ± 0.04	0.86 ± 0.04	0.86 ± 0.04	0.85 ± 0.07	0.93 ± 0.04*
Fecal bile acids (μmol/g)	16.50 ± 0.17	17.38 ± 0.45	27.32 ± 0.67*	31.71 ± 1.43$^{\#}$	61.60 ± 1.85$^{*\#}$	71.18 ± 2.08$^{\$}$
Fecal neutral sterol (μmol/g)	0.99 ± 0.05	1.09 ± 0.33	94.1 ± 1.7*	97.9 ± 0.9	109 ± 5$^{*\#}$	107 ± 2

Values are means ± SE, n=4. * Significantly different from the N group; $^{\#}$ significantly different from the C group; $^{\$}$ significantly different from the CB group.

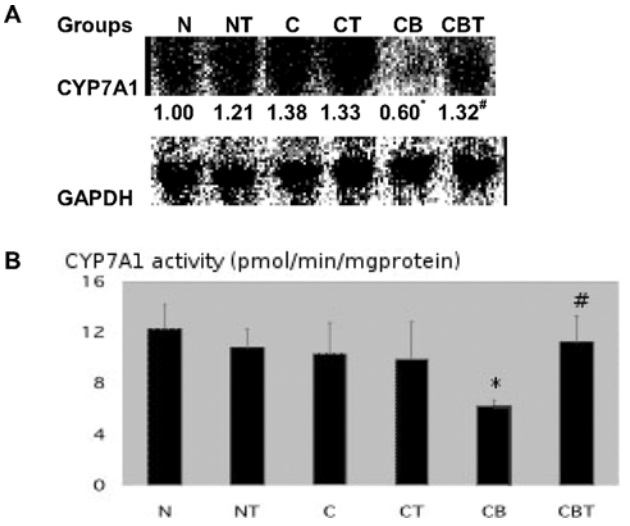

Figure 1. Effects of diets on mRNA level (A) and activity (B) of CYP7A1 in mice. After animals had been fed with respective diets for 7 days, the livers were obtained at 23:00 in the dark cycle for CYP7A1 mRNA level and activity detection. mRNA expression was quantitated relative to GAPDH by Northern blot. *: significantly different from the N group; $^{\#}$: significant different from the CB group ($p<0.05$).

The mRNA level and activity of CYP7A1 were significantly decreased in the CB group compared to that of N group and both of them were notably improved by taurine supplementation in the CBT group (Fig. 1). No change was observed in CYP7A1 activity in the C group comparing to that of N group although its mRNA level tended to increase, and taurine did not show any effect in both these groups.

Fig. 2A shows the Northern blot results of nuclear receptors related to the up-regulation of CYP7A1 gene expression. All of them, LXR, LRH, and HNF4α, were not changed by the diets, neither were cholesterol, cholic acid, and taurine. FXR and SHP, which are reported to down-regulate CYP7A1 gene expression, were not affected by the diets, although SHP in the C and CT groups tended to decrease (Fig. 2B). These data suggest that taurine may not directly affect mRNA levels of the above nuclear receptors.

The mRNA levels of liver ABCG5 were increased in the C and CT groups compared to that in the N group, and further improved in the CB and CBT groups, but not mediated by taurine (Fig. 3A). Similarly to ABCG5, ABCG8 was also not mediated by taurine. No differences were observed on BSEP among the six groups. These data suggest that the excretion of cholesterol and bile acids from the liver to bile was not affected by taurine supplementation.

The mRNA levels of jejunum ABCG5 and ABCG8 (Fig. 3B) were significantly increased in the C and CB groups compared to that in the N group, and no changes were observed by taurine supplementation. This is consistent with the results on fecal neutral sterol excretion, and suggests that taurine seems to have no effects on cholesterol excretion from the intestine.

Fig. 3C shows the Northern blot results of factors related to re-absorption of bile acids from the ileum. I-BABP was not affected by the diets. I-BAT tended to be reduced accompanying with the addition of cholesterol to the diets. A decreased tendency and an increased tendency were observed in the CT and CBT groups, respectively, in contrast to their own control groups, C and CB groups, respectively.

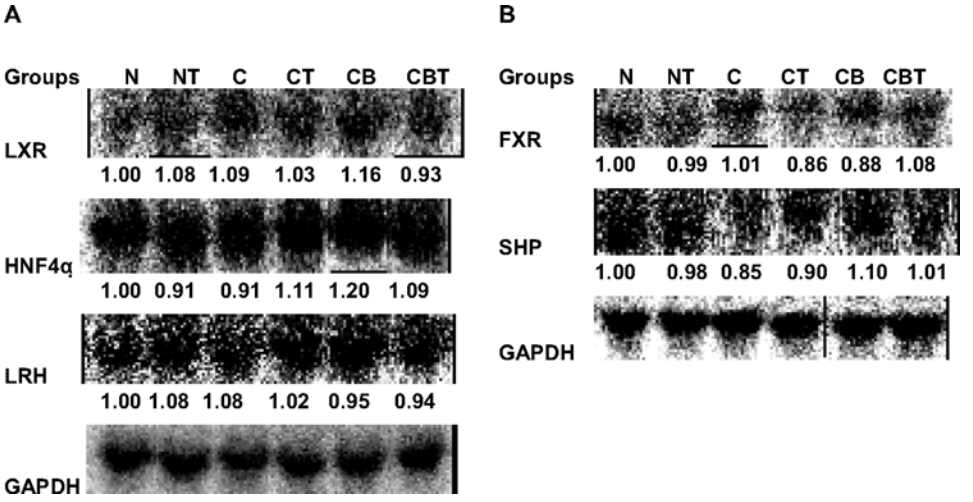

Figure 2. Northern blot results of the nuclear receptors related to up- (A) or down- (B) regulation of CYP7A1 gene expression. The mice were fed with respective diets for one week. mRNA expression was quantitated relative to GAPDH.

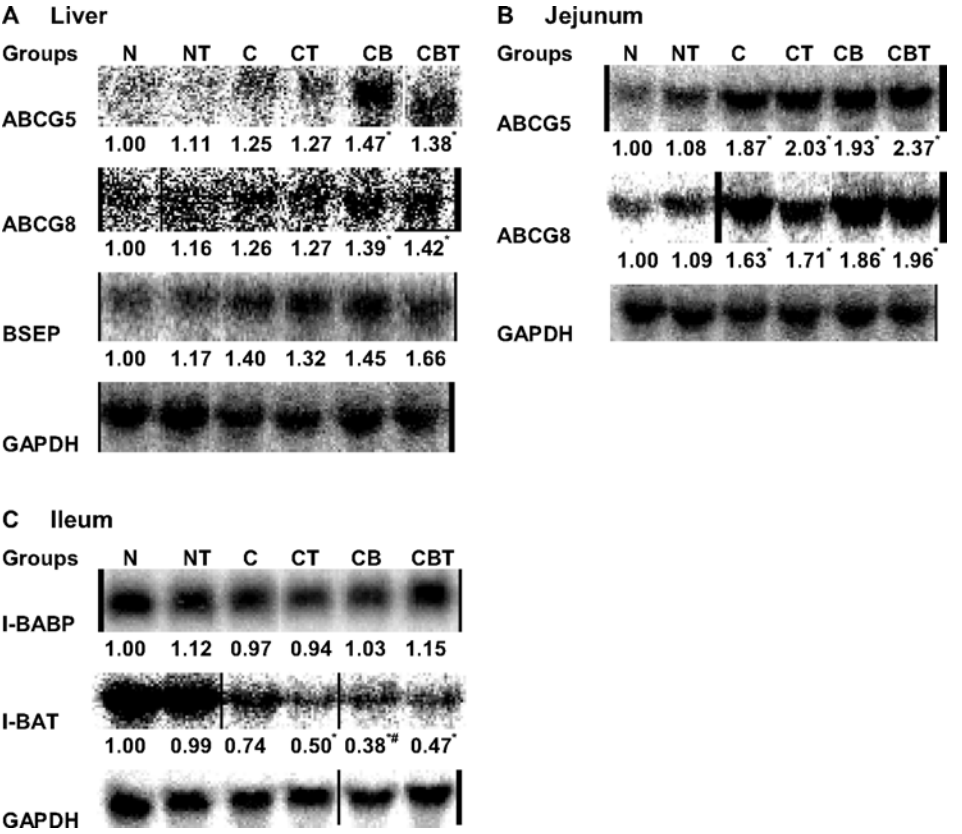

Figure 3. Northern blot results of the factors related to the excretion of cholesterol and bile acids from the liver to bile (A), the excretion of cholesterol from jejunum (B) and the re-absorption of bile acids from ileum (C). The mice were fed with the respective diets for one week. mRNA expression was quantitated relative to GAPDH. *: significantly different the from N group; #: significantly different from the C group ($p<0.05$).

4. DISCUSSION

CYP7A1 is the rate-limiting enzyme in the classic pathway, the main pathway in bile acids biosynthesis. During the past years, several nuclear receptors have been identified to regulate CYP7A1 gene expression. Both LXRα and FXR, which form heterodimers with a common partner RXR, have critical regulatory functions by virtue of their roles as sensors of dietary cholesterol and intracellular bile acids, respectively (Stroup *et al.,* 1997, Sinal *et al.,* 2000). HNF4α and LRH are the two orphan nuclear receptors that normally bind to the responsive element in the CYP7A1 promoter and activate its transcription (Crestani *et al.,* 1998; Nitta *et al.,* 1999), whereas SHP, induced by bile acid-activated FXR, interacts with HNF4α and LRH and then suppresses CYP7A1 gene transcription (Goodwin *et al.,* 2000). Our results show that no changes in the mRNA levels of the above nuclear receptors were observed by diet treatment, although the mRNA level of CYP7A1 was significantly decreased by cholic acid feeding and then notably improved by taurine supplementation. These data suggest that (1) although there

were no changes in the mRNA level, FXR is indeed activated by cholic acid feeding and then functions as a down-regulator to CYP7A1, (2) taurine may inhibit or alleviate activation of FXR via an indirect pathway but it is not a ligand for FXR, and (3) taurine may also activate LXRα, HNF4α or LRH by an indirect route because it is neither a probable ligand for them.

There are two major mechanisms of bile acids on feedback regulation of CYP7A1 gene expression, SHP-dependent one, as described above, and SHP-independent one (Chiang, 2003). In the SHP-independent feedback pathway, bile acids can also down-regulate CYP7A1 gene expression by activating c-Jun (Gupta *et al.,* 2001). Bile acids activate the mitogen-activated protein kinase (MAPK) signaling pathway including c-Jun N-terminal kinase (JNK). Subsequently, c-Jun is phosphorylated by JNK. c-Jun is also supposed to inhibit CYP7A1 gene expression directly or through interacting with LRH, although SHP has already been demonstrated being a direct target of activated c-Jun (Gupta *et al.,* 2001; Chiang, 2003). Therefore, according to the reports that taurine depletion stimulates phosphorylation of some proteins in the rat heart, it is conceivable that taurine may restrain the activation of c-Jun caused by bile acids through inhibiting phosphorylation and then improve the gene expression (Lombardini, 1996).

Decrease of *de novo* synthesis, increase of degradation and large excretion of cholesterol are three auto-regulation mechanisms to maintain cholesterol homeostasis when high cholesterol is present in the diet. ABCG5 and ABCG8 have functions to mediate the excretion of cholesterol not only from the liver to bile, but also from the intestine to the instestinal lumen (Berge *et al.,* 2000). In the present experiment, although the induction of ABCG5 and ABCG8 by high cholesterol diets was observed, taurine seems to have no effect on inducing their expression. This is consistent with the result of fecal neutral sterol excretion.

Biosynthesis, excretion to feces and re-absorption from the ileum are three important sides in bile acid homeostasis in the body. Except re-absorption, the other two points have been demonstrated to be increased by taurine. BSEP and I-BABP are FXR target genes which are responsible for exporting bile acids to bile and facilitating bile acid uptake in the apical membrane, respectively (Grober *et al.,* 1999; Ananthanarayanan *et al.,* 2001), whereas I-BAT, located in the brush border membrane of ileocytes, is the key factor that mediates bile acid re-absorption from the intestine (Zhang *et al.,* 2002). Once taken up by I-BAT, bile acids are bound to I-BABP. Our results show that there were no changes in BSEP and I-BABP among the six groups, but the mRNA level of I-BAT tended to decrease and increase in the CT and CBT groups, respectively, when compared to their own controls, the C and CB groups. The decrease in the I-BAT mRNA level in the CT group when compared to that in the C group may be the key point to explain the reduction of serum and liver cholesterol (data not shown), because CYP7A1 activity was not improved in this case. According to the results on fecal bile acid excretion and I-BAT mRNA level, it is conceivable that taurine lowers serum and liver cholesterol through improving excretion and suppressing re-absorption of bile acids when taking high cholesterol diet without exogenous cholic acid.

In the CB group, with the addition of exogenous cholic acid to the high cholesterol diet, the I-BAT mRNA level was significantly decreased when compared to the C group. This suggests that bile acid uptake in the ileum is inevitably reduced in the presence of large amount of exogenous cholic acid. Contrary to the case of the C and CT groups, the I-BAT mRNA level in the CBT group tended to increase when compared to that in the CB group. This suggests that taurine seems to have no effect on repressing bile acid re-

absorption in the case of cholic acid feeding. Meanwhile, as taurine notably improved the CYP7A1 mRNA level only upon CB diet feeding, this also suggests that bile acids may be necessary for taurine to induce CYP7A1 gene expression under this special physiological condition although the mechanism is unclear at present. In other words, taurine induces CYP7A1 mRNA only when a large amount of dietary cholesterol is assimilated because of the effects of cholic acid on cholesterol uptake.

In brief, our results suggest that (1) taurine may inhibit the SHP-dependent mechanism by alleviating activation of FXR, blunt the JNK/c-Jun pathway by suppressing phosphorylation, or indirectly activate LXRα, HNF4α or LRH in order to improve CYP7A1 gene expression; (2) taurine has no effect on promoting fecal neutral sterol excretion; and (3) taurine tends to increase I-BAT gene expression in the case of high cholesterol+bile acid diet feeding, whereas it decreases I-BAT gene expression in the case of cholesterol feeding without exogenous cholic acid. This suggests that CYP7A1 is not repressed even when much bile acids are re-absorpted from the ileum in the high cholesterol+bile acid+taurine group compared with the high cholesterol+bile acid group, because taurine has blocked the pathway of inhibition of CYP7A1 caused by cholic acid. More studies are necessary to reveal the mechanism of cholesterol-lowering effect of taurine.

5. ACKNOWLEDGMENTS

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COMPARISON OF THE EFFECTS OF TAURINE WITH THOSE OF RELATED SULFUR-CONTAINING COMPOUNDS ON PYRIDOXAL-INDUCED ADRENOMEDULLARY CATECHOLAMINE RELEASE AND GLYCOGENOLYSIS IN THE RAT

Cesar A. Lau-Cam and Jagir P. Patel*

1. ABSTRACT

Taurine (2-aminoethanesulfonic acid) is known to attenuate the release of adrenomedullary catecholamines and ensuing hepatic glycogenolysis and hyperglycemia induced by pyridoxal in the rat. Using this animal model, the present study was undertaken to assess the impact that simple structural modifications of the taurine molecule might have on its antagonistic actions against PL. Removal of the amino group (ethanesulfonic acid) or shortening the carbon chain by one methylene (2-aminomethanesulfonic acid) raised the protective actions of taurine. While N-alkylation (N-methyltaurine) or replacement of the amino group by hydroxyl (isethionic acid) had a lowering effect, substituting a sulfhydryl group (2-mercaptoethanesulfonic acid) for the 2-amino group abolished all antagonistic properties associated with taurine. The sulfinic acid analog (hypotaurine) was equipotent with taurine, but the carboxylate isostere (β-alanine) was inactive. Propranolol, a nonspecific β-adrenoceptor antagonist, enhanced the antiglycogenolytic effect of taurine and of all structurally related compounds capable of attenuating the outflow of adrenal catecholamines elicited by pyridoxal.

2. INTRODUCTION

At doses at least 100-fold above the required daily allowance, vitamin B_6 vitamers manifest properties that are totally unrelated to their recognized biological functions in mammalian systems. While sensory and motor neuropathy (Schaumburg *et al*., 1983;

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Taurine 6
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Friedman *et al*., 1986; Waterston and Gilligan, 1987; Morra *et al*., 1993) has been linked to the consumption of doses of pyridoxine in excess of 100 mg/day and have been the subject of extensive experimentation in humans (Parry and Bredesen, 1985; Dalton and Dalton, 1987) and laboratory animals (Krinke *et al*., 1981; Schaeppi and Krinke, 1982, 1985; Xu *et al*., 1989; Wietholter *et al*., 1990; Perry *et al*., 2004), deleterious effects such as hepatotoxicity have remained virtually unexplored. In the course of evaluating the adverse effects of pyridoxal, in doses as high as 850 mg/kg, and of pyridoxine, in as much as1200 mg/kg, in rats, histopathological examination of liver sections unexpectedly revealed a complete absence of glycogen granules in the hepatocytes (Kendall, 1984). Subsequent systematic investigation of the factor responsible for this finding uncovered a sympathoadrenal mechanism leading to the release of adrenomedullary catecholamines (CATs) for interaction with hepatic β-adrenoceptors, activation of glycogen phosphorylase and eventual glycogenolysis (Kendall, 1984; Lau-Cam *et al*., 1991).

CATs are released into the circulation as a result of the presentation of the appropriate physiologic stimulus (i.e., stress, exercise, insulin) to the sympathoadrenal apparatus (Wurtman, 1996). As a result, CATs enter the blood in short-lived spurts and are eventually excreted in the urine. Circulating CATs originate from two main sources, the chromaffin cells localized in the adrenal medulla and sympathetic nerve endings present in almost all tissue. Since bilateral adrenalectomy causes a marked and rapid fall (by more than 80 per cent) in urinary epinephrine levels with little change in NE excretion, one can safely assume that the bulk of circulating epinephrine is of adrenal origin (Wurtman, 1996). When rats were exposed to immobilized cold stress (as an immersion in 30°C water for 3 h), Nakagawa and Kuriyama (1975) verified that the epinephrine content in the adrenal gland and noradrenaline content in the brain stem were reduced drastically together with a significant increase in blood sugar. Oral administration of taurine (4-7 g/day) for 3 days prevented the stress-induced decline of epinephrine in the adrenal gland and the rise in blood glucose, but it did not have a significant effect in adrenal tyrosine hydroxylase activity, dopamine-β-hydroxylase activity and the turnover rate of epinephrine. The regulatory mechanism of action of TAU was interpreted as being related to its ability to reduce epinephrine output from the adrenal gland, possibly by stabilizing the membrane of the chromaffin granules, by increasing intragranular calcium through an increase in affinity, or by both actions (Nakagawa and Kuriyama, 1975; Kuriyama and Nakagawa, 1976).

The present study was carried out in the rat to: (a) examine the effect of TAU on the release of CATs elicited by a high, acute, dose of PL, (b) compare the potency of TAU with that of sulfur-containing compounds representing analogs and homologs of TAU, (c) establish structure-activity relationships for TAU, and (d) determine whether TAU and its congeners share a common mechanism of action on PL-related biochemical alterations. The test compounds were compared with TAU in terms of the plasma total CATs, plasma glucose and hepatic glycogen levels at 30 min post-PL.

3. MATERIALS AND METHODS

3.1. Materials

Pyridoxal hydrochloride (PL), taurine (TAU), aminomethanesulfonic acid (AMSA), hypotaurine (HYTAU), isethionic acid (ISA) and propranolol hydrochloride (PRO) were

purchased from Sigma Chemical Company, St. Louis, MO. β-Alanine (BALA) was from Arcos Organics, New Jersey, ethanesulfonic acid sodium salt (ESA) was from Aldrich Chemical Company, Milwaukee, WI, N-methyltaurine (MTAU) from Pfaltz & Bauer, Waterbury, CT, and 2-mercaptoethanesulfonic acid (MESA) from ICN Biomedicals, Aurora, OH.

3.2. Animals

All experiments were conducted using groups of 5 male Sprague-Dawley rats, 200-250 g in weight, purchased from Taconics (Germantown, NY) and housed in groups of 5 in plastic cages, in a room maintained at a constant temperature of 21 ± 3˚C, constant humidity, and a normal 12 h light-dark cycle. During an acclimation period of at least 3 days, the rats were fed a commercial rat diet (Purina Lab Chow™, Ralston-Purina Co., St. Louis, MO) and water ad libitum. The animals were deprived of food for 12 h, overnight, before an experiment.

3.3. Treatments

The various test compounds were dissolved in distilled water and administered by the intraperitoneal route. The dose of a sulfur-containing compound was 2.4 mmol/kg, given in two divided doses 30 min apart, followed 30 min later by a bolus of 300 mg/kg dose of PL. In experiments with PRO, this compound was given at the dose of 2 mg/kg, 30 min before a dose of TAU.

3.4. Samples and Assays

Blood samples were collected at 30 min post-PL by the orbital sinus technique of Riley (1960) into microtubes containing a small amount of NaF-Na_2EDTA, mixed well, and centrifuged at 2,500 rpm for 10 min to isolate the plasma fraction. The samples were kept on ice pending their analysis for glucose levels or frozen until their analysis for CATs. Plasma glucose was measured using a commercially available kit (Procedure No. 510 from Sigma Chemical Co., St. Louis, MO). Plasma CATs (epinephrine, norepinephrine, dopamine) were measured by a HPLC method with electrochemical detection as described by Williams *et al*. (1985). The test samples were purified by the method of Wang *et al*. (1999), and analyzed in the presence of 3,4-dihydroxy-benzylamine serving as an internal standard. Livers were removed by the freeze-clamp technique of Wollenberger *et al*. (1960) from anesthetized rats (sodium phenobarbital), immediately after collecting a blood sample, and were kept at -70˚C until needed for the assay of hepatic glycogen according to Keppler and Decker (1974). A sample for this purpose was prepared by homogenizing a portion of frozen liver with ice-cold 0.6 M $HClO_4$ (1 g/5 ml), neutralizing an aliquot of the homogenate (0.2 ml) with 1 M $KHCO_3$ (0.1 ml), and hydrolyzing the glycogen by an incubation (40˚C) with a solution of amyloglucosidase (30-60 units/mg of protein, from *Aspergillus niger*) containing 45 units/ml (2 ml). Following centrifugation of the mixture at 3,000 rpm for 10 min, the glucose present in the supernatant was measured in described for the plasma samples.

3.5. Statistical Analysis of the Data

The experimental results are reported as the mean ± SEM for n = 5. They were analyzed using a commercially available software program (SPSS™ Version 12.0, John Wiley & Sons, New York). Differences between groups were considered to be statistically significant at $p<0.05$ by unpaired Student's t-test and one-way ANOVA followed by Neumann-Keuls *post-hoc* test.

4. RESULTS

The dose of PL (300 mg/kg) used in all the experiments was determined from a dose-response study (elevation of plasma total CATs and plasma glucose and depletion of hepatic glycogen) with graded doses in the 75-450 mg/kg range (Fig. 1). A second study with two doses of TAU (1.2 and 2.4 mM/kg) found this compound to attenuate the release of adrenal CATs into the circulation in a dose-related manner (Fig. 2) and to exert a greater effect when its higher dose was given in two equipotent portions and 30 min apart rather than as a bolus dose ($p<0.001$ for both dosing schedules).

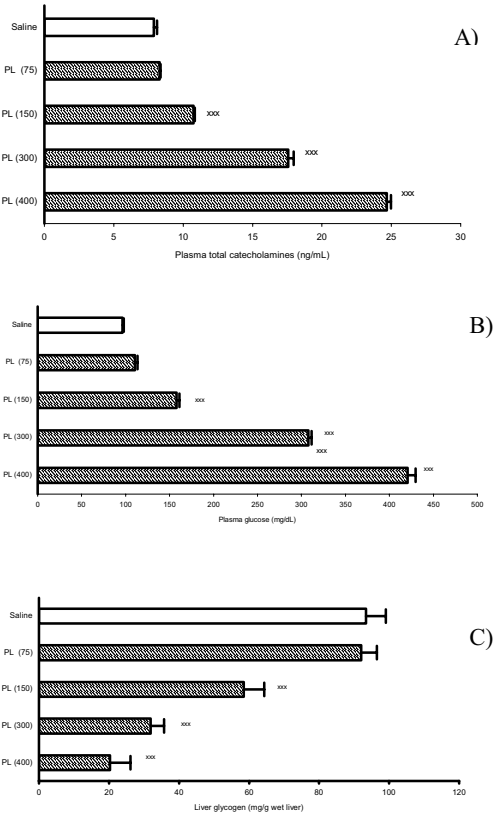

Figure 1. The effect of different doses of PL (75-450 mg/kg) on: (A) plasma CATs, (B) plasma glucose and (C) liver glycogen. Values are given as mean ± SEM for n = 5. Statistical comparisons vs. saline: ***$p<0.001$.

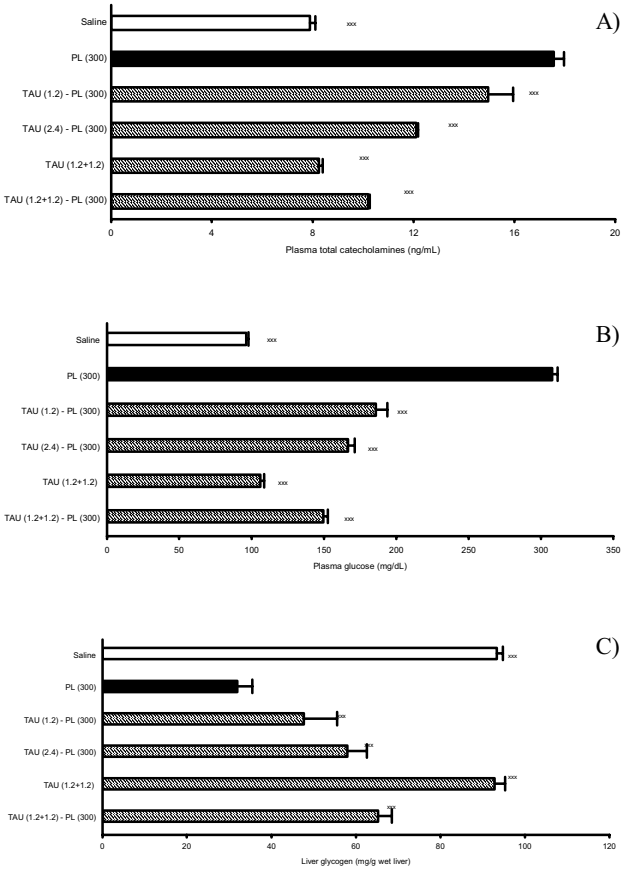

Figure 2. Effect of various doses (1.2, 2.4, 1.2+1.2 mmol/kg) and dosing schedules (bolus, divided doses) of TAU on: (A) plasma CATs, (B) plasma glucose and (C) liver glycogen of rats treated with PL (300 mg/kg). Values are mean ± SEM for n = 5. Statistical comparisons vs. PL: *** $p<0.001$.

The effects of the various structural modifications of TAU on PL-related biochemical alterations are graphically shown in Table 1. The role played by the amino group was investigated with the deaminated analog ESA. This compound attenuated the PL-mediated release of CATs from the adrenal medulla to an extent equivalent to that attained with TAU, and was insignificantly better than TAU in preventing the accompanying alterations in plasma glucose and hepatic glycogen. A similar trend of results was obtained with AMSA, the immediate lower homolog of TAU. In contrast, substituting the amino functionality of TAU by a hydroxyl (i.e., ISA) or sulfhydryl (i.e., MESA) group led to a significant loss ($p<0.001$) in protecting ability relative to the parent compound. Furthermore, N-monomethylation of TAU resulted in a compound (MTAU) with effects that approximated those of PL itself. The sulfinic analog, HYTAU, was equipotent with TAU against all alterations brought about by PL, but the carboxyl one, BALA, was as weak as ISA and MTAU.

Table 1. Effect of TAU and structurally related sulfur containing compounds on the plasma total CATs, plasma glucose and liver glycogen 30 min after a treatment with PL (300 mg/kg)[a,b,c]

Treatment	Plasma total CATs (ng/ml)	Plasma glucose (mg/ml)	Liver glycogen (mg/g wet liver)
Saline	7.89 ± 0.22***	96.35 ± 1.43***	93.40 ± 5.69***
PL	17.56 ± 0.41	307.87 ± 3.58	31.94 ± 3.79
AMSA + PL	10.58 ± 0.54***	134.36 ± 4.61***	67.42 ± 2.75***
BALA + PL	15.52 ± 0.13**	282.49 ± 4.01**	34.86 ± 2.69*
ESA + PL	10.42 ± 0.25***	131.89 ± 4.26***	68.29 ± 3.41***
HYTAU +PL	10.63 ± 0.21***	147.32 ± 2.51***	63.55 ± 5.78***
ISA + PL	15.05 ± 0.34**	181.57 ± 3.3.50***	46.85 ± 2.78**
MESA + PL	18.41 ± 1.12*	296.48 ± 6.28	32.86 ± 2.06
MTAU + PL	15.05 ± 0.21**	266.96 ± 3.89**	38.41 ± 2.57*
TAU + PL	10.20 ± 0.06***	149.53 ± 3.25***	65.21 ± 6.90***

[a] All test compounds were given at a dose of 1.2 mmol/kg + 1.2 mmol/kg 30 min apart and 30 min before PL. [b] Results are given as the mean ± SEM for groups of 5 rats each. [c] Statistical comparisons: *p<0.05, **p<0.01, ***p<0.001 vs. PL.

Table 2. Effect of PRO plus TAU or a structurally related sulfur containing compounds on the plasma total CATs, plasma glucose and liver glycogen 30 min after a treatment with PL (300 mg/kg)[a,b,c]

Treatment	Plasma total CATs (ng/ml)	Plasma glucose (mg/ml)	Liver glycogen (mg/g wet liver)
Saline	7.89 ± 0.22***	96.35 ± 1.43***	93.40 ± 5.69***
PL	17.56 ± 0.41	307.87 ± 3.58	31.94 ± 3.79
TAU+PL	10.20 ± 0.06***	149.53 ± 3.25***	65.21 ± 6.90***
PRO	8.09 ± 0.12***	117.30 ± 2.07***,†††	94.76 ± 5.02***,†††
PRO+AMSA+PL	10.87 ± 0.36***	84.17 ± 2.14***,†††	91.88 ± 5.61***,†††
PRO+ESA+PL	11.37 ± 0.37***	85.19 ± 3.17***,†††	95.49 ± 3.16***,†††
PRO+HYTAU+PL	10.43 ± 0.37***	85.79 ± 4.26***,†††	94.38 ± 4.38***,†††
PRO+ TAU+PL	10.57 ± 0.24***	81.05 ± 2.39***,†††	100.71 ± 4.02***,†††

[a] The dose of PRO was 2 mg/kg. The dose of the test compound was 1.2 mmol/kg + 1.2 mmol/kg, given 30 min apart and 30 min before PL. [b] Results are given as the mean ± SEM for groups of 5 rats each. [c] Statistical comparisons: ***p<0.001 vs. PL; †††p<0.001 vs. TAU + PL..

Treating rats with PRO and TAU ahead of PL enhanced the protective action of TAU on the hepatic glycogen (p<0.001) but without altering the extent of CATs release seen with TAU alone (Table 2). To verify whether or not those sulfur compounds with a demonstrable attenuating action on PL-related CAT release from the adrenal gland (i.e., TAU, ESA, AMSA, HYTAU) are operating by a common mechanism of action, a study

in which PRO was administered prior to a sulfur-containing compound and PL was also conducted. From the results presented in Table 2, it is apparent that while this pharmacological antagonist did not modify the levels of CATs found in the plasma of rats treated with a protective sulfur compound plus PL, it, however, markedly enhanced their ability to preserve the hepatic glycogen (by 41-54%, $p<0.001$) and to lower the elevations in plasma glucose (by 43-46%, $p<0.001$) relative to TAU alone ($p<0.001$).

5. DISCUSSION

In spite of its small molecular size and limited number of reactive functionalities, TAU is a compound that has been the subject of numerous structure-activity correlation studies. The great interest on the effects that structure variation may have on the properties of this β-amino acid may stem from the ready availability of a large number of structural candidates with potential for evaluation and from the wide range of biological properties to which such correlations may apply. From a review of the current scientific literature, it appears that simple structural modifications like those evaluated here may, under a different set of conditions, exert effects that are neither constant in magnitude and direction nor predictable from results relevant to a different biological role. In the present study, the amino group is found not to be essential to the attenuating effect of TAU on the release of adrenal CATs by a chemical challenge since the deaminated analog, ESA, demonstrated an insignificantly greater attenuating effect than TAU. That the action of TAU on the adrenal gland is centered on its sulfonate rather than on its amino group is inferred from the observation that BALA the carboxylate isostere of TAU was without effect. The same type of reasoning has been used to explain the binding of TAU and HYTAU to insulin receptors and the lack of displacing effect of TAU from the agonist-receptor complex by BALA (Maturo and Kulakowski, 1988). The close dependency of the observed activity on the experimental conditions is exemplified by the results of an earlier work in this laboratory in which ESA was found be an antioxidant as good as TAU in isolated erythrocytes exposed to hydrogen peroxide (Pokhrel and Lau-Cam, 2000a); but it became significantly less protective than TAU when the oxidant was changed to phenylhydrazine (Pokhrel and Lau-Cam, 2000b). Similarly, a comparison for antiarrhythmic activity in a dog receiving a large dose of digitalis found TAU and 3-propanesulfonic acid to be active but not ESA and ethanedisulfonic acid (Welty *et al*., 1976).

Although the carbon chain length appears to play an important role in many of the biological activities of TAU, its determining role is susceptible to an unpredictable variability. Thus, since in this work AMSA, the lower homolog of TAU was equipotent with ESA and TAU in antagonizing PL-related effects, one would expect an effective interaction between TAU or a structural modification of TAU and a target organ or cell to occur when the anionic and cationic groups are separated by one to two methylene groups. This line of reasoning is supported by the results of a study attempting to characterize the uptake of TAU into rat lung slices and which found HYPO and BALA, but not homotaurine, to be uptaken (Lewis *et al*., 1990). However, there is also evidence to indicate that while TAU promotes calcium uptake across a retinal vesicular preparation AMSA inhibits the process (Lombardini, 1985). Similarly, by comparing the extent of binding of a series of compounds structurally related to TAU to TAU receptors on olfactory cells of the spiny lobster it was determined that the ability to stimulate the

receptors was highest when the amino and sulfonate groups were separated by two carbons, lacking in compounds with only one carbon or without the amino group, and to decrease as a function of the distance of separation beyond a two-carbon chain (Gleeson *et al*., 1987). The same conclusion was reached in a study in the rat that found the cerebroventricular injection of homotaurine (HTAU) to elicit a more intense hypothermia and depression of motor behavior than TAU, and AMSA to exhibit only a weak activity (Palmi *et al*., 1987). Yet, in experiments with rat liver mitochondrion, while TAU promoted the uptake of calcium and uncoupled oxidative phosphorylation, neither. HTAU nor ISA produced a significant effect (Palmi *et al*., 1996).

Unlike other structural modifications of TAU, substitution of the amino functionality for an unprotonated hydroxyl (like in ISA) or sulfhydryl (like in MESA) group are usually found to negatively affect the biological actions of TAU, more so with the latter than with the former modification. The present results for the attenuation of PL-induced adrenal secretion of CATs by ISA and MESA are in line with these conclusions. Furthermore, when evaluated for antioxidant action, while ISA was about half-as potent as TAU, MESA was totally inactive regardless of the oxidant present (Pokhrel and Lau-Cam, 2000a, 2000b). When tested as a mediator of ATP-dependent calcium ion uptake in the rat retina at low calcium concentrations, N,N-dimethyltaurine and TAU stimulated the process but HTAU and ISA were inactive (Lombardini and Liebowitz, 1990).

It would appear that although the amino group is not indispensable for some of the biological activity of TAU, when present it can exert a strong influence on the sulfonate group. One possibility would be by entering into zwitterion formation, a chemical state that may allow TAU to intercalate by ion pair formation into head groups of membrane acylphosphoglycerides to modify the properties of membranes like that of adrenal chromaffin cells not to permit the release of CATs (Huxtable, 1989). Another possibility, which may apply to situations where TAU interacts with surface receptors, is that in an zwitterion state TAU is able to interact with recognition sites on the receptor that bear charges opposite to those of the quaternary nitrogen and sulfonate anion (Sgaragli *et al*., 1996). This ability of TAU may be lost when a hydroxyl substituent is present on the β-carbon (like in ISA) or hindered when the amino group is mono (MTAU) or dialkylated (N,N-dimethyltaurine). The extreme variability in physiological effects that can be associated with the amino end of the TAU molecule is clearly exemplified by an experiment in rabbits in which the brain intraventricular infusion of TAU, MTAU and N,N-dimethyltaurine led to hypothermia, hypothermia or hyperthermia depending on the dose, and hyperthermia, respectively (Sgaragli *et al*., 1996). In contrast, replacing the sulfonate group for sulfinate (like in HYTAU) preserved all of the biological characteristics of TAU. Hence, for significant biological activity to occur in analogs and homologs of TAU the simultaneous presence of an unsubstituted amine and a sulfonate group or of an unsubstituted amine and a sulfinate group may be required (Petegnief *et al*., 1995).

6. CONCLUSIONS

Modifications to the structure of TAU such as a shorter carbon chain, substitution of sulfinate for sulfonate group and deamination are found to preserve the effects of TAU on PL-induced adrenal CATs secretion and hepatic glycogenolysis in the rat. In contrast, N-methylation, replacement of amino by sulhydryl or hydroxyl or of a carboxyl for a

sulfonate functionality either weakens or abolishes the antagonistic actions of TAU. Like the parent compound, the actions of these structural modifications appear to be limited to the adrenal gland. In general, the present results follow the trend of structure-activity relationships documented for other biological actions of TAU.

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ACCUMULATION OF TAURINE IN TUMOR AND INFLAMMATORY LESIONS

Chaekyun Kim*

1. INTRODUCTION

Taurine is an abundant amino acid in mammalian tissues including inflammatory cells where it acts as a trap for toxic hypochlorous acid ($HOCl/OCl^-$). Although differences exist among cells and species, the intracellular concentration of taurine in mammalian tissues is millimolar concentrations (Fukuda *et al.,* 1982). In particular, taurine is found in high concentrations in the brain, liver, lung, kidney, muscle, intestine, heart and adrenal gland (Jhiang *et al.,* 1993; Sturman, 1993). A close correlation between endogenous taurine concentrations and bio-distribution of exogenously injected taurine, except brain and lung, was reported (Shimada *et al.,* 1984; Kim *et al.,* 1998). Injected taurine was mainly accumulated in the liver, kidney, intestine, eye, hypophysis, thymus, stomach and adrenal glands.

Taurine protects tissue from damage resulting from overt inflammatory reactions by reacting with $HOCl/OCl^-$ to form taurine chloramine, which protects cells by regulating the production of many pro-inflammatory mediators (Park *et al.,* 1995, 1998; Kim *et al.,* 1996; Marcinkiewicz *et al.,* 1998; Barua *et al.,* 2001). Thus, the accumulation of taurine in inflammatory lesions may be a prerequisite for its anti-inflammatory action (Kim *et al.,* 1998). Inflammation lesion and cancer tissue are characterized by increased glucose and amino acids. Because taurine is the most abundant amino acid in the plasma and leukocytes, taurine may accumulate in inflammation lesion and cancer tissue. However, distribution of taurine in inflammation or cancer tissue is not known.

This study was designed based on the report that the affinity of the taurine transporter is significantly different in different cell lines (Ganapathy and Leibach, 1994). Although taurine is charged, it can be transported into cells by the sodium- and chloride-dependent taurine transporter. Taurine transporter has been cloned from various mammalian cells, and the genes are highly homologous (Liu *et al.,* 1992; Jhiang *et al.,* 1993; Ramamoorthy *et al.,* 1993). The disruption of taurine transporter gene in the mouse produced markedly decreased taurine levels, especially in the plasma, liver,

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Taurine 6
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kidney, eye, heart and skeletal muscle (Heller-Stilb *et al.,* 2002). We hypothesized that accumulation of taurine in tumor and inflammatory lesions may different. We determined the distribution of taurine in ICR mice having tumor, chronic muscle inflammation, or both.

2. MATERIALS AND METHODS

2.1. Experimental Tumor and Inflammation Models

Male ICR mice (30-35 g), supplied from Seoul National University (Korea), were subcutaneously inoculated into the left groin with 0.1 ml Sarcoma 180 cells (1 x 10^6 cells/ml) to induce tumor, and intramuscularly injected into the right thigh with 0.1 ml turpentine oil to induce inflammation. On the 7th day post inoculation, typical sarcoma and chronic inflammation were observed.

2.2. Bio-Distribution of Taurine in Mice Having Tumor and/or Inflammation

The distribution study was performed using the mice having tumor, inflammation, or tumor plus inflammation after 7 days of injection. Groups of four or five mice were injected intravenously through the tail vain with 1 μCi of [^{3}H]taurine (Du Pont NEN, Boston, MA) in 0.1 ml of saline and were sacrificed 1 h after injection. Tissues of interest were removed, weighed and dissolved overnight in 1 ml of Soluene 350 (Packard, Downers Grove, IL). Radioactivity was counted with 10 ml of liquid scintillation solution (Ultima Gold XR, Packard) in a liquid scintillation analyzer (RPI-CAPB, Packard). The percent of injected dose (% ID) per gram of tissue and % ID per organ were calculated by comparing the activity in each sample with an injection standard of appropriate count rate.

2.3. Statistical Analysis

The two-tailed Student's t-test (paired) was performed using Microsoft Excel (Redmond, WA). Data are expressed as means ± SD and a p value <0.05 was considered significant.

3. RESULTS AND DISCUSSION

Taurine is present as a free amino acid and conjugate with bile acid in the liver (Liu *et al.,* 1992; Sturman, 1993). The injected taurine is rapidly taken up by the kidney, liver, spleen and bone marrow whereas the uptake is considerably slow in the heart and muscle (Huxtable, 1981). The rate of turnover of taurine was tissue-dependent and the rate of cellular uptake and the retention varied with cell type or organ. No metabolism of injected taurine in the organs was reported and taurine showed a high reactivity for glucose and acetaldehyde (Boquet and Fromageot, 1965). We compared the tissue distribution of injected taurine in mice having tumor, chronic inflammation and tumor plus inflammation.

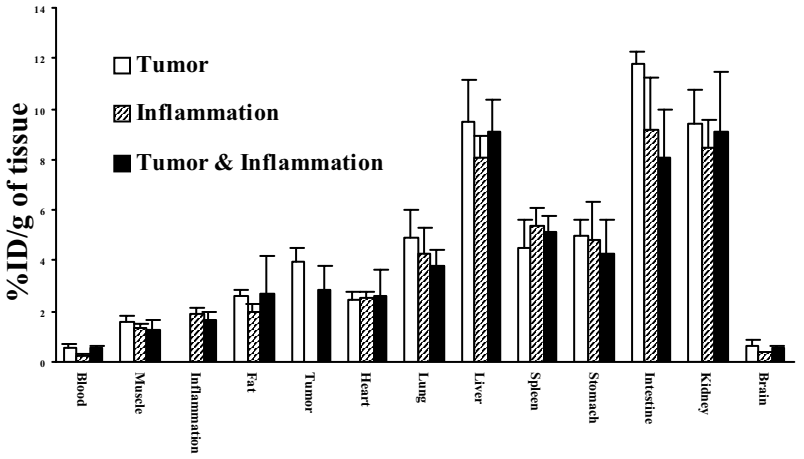

Figure 1. Distributions of [^{3}H]taurine in ICR mice having tumor and chronic inflammatory lesions at 1 h post injection. The data are expressed in percent of injected dose (% ID) per gram of tissue (n=5, inflammation group and inflammation plus tumor group; n=4, tumor group).

Tissue distribution was determined at one hour after intravenous injection of 1 μCi of [^{3}H]taurine. It was distributed without influencing the overall distribution in the control mice (not shown). The overall distribution patterns were similar between groups (Fig. 1). Significant accumulation of [^{3}H]taurine was observed in tumors (tumor/blood = 8.1 and 6.6, respectively) with the tumor to muscle ratio of 2.6 and 2.4, respectively, in tumor and tumor plus inflammation groups. The accumulation in inflammatory lesions (inflammation/blood = 6.4 and 3.2, respectively) was observed with the inflammation to muscle ratio 1.4 both in inflammation and tumor plus inflammation groups (Table 1). The elevated taurine in inflammatory lesions may act as a scavenger of toxic HOCl/OCl$^-$ and may be attributed to the anti-inflammatory role of taurine. Vercer *et al.* (1998) reported that taurine levels are significantly low in muscles of colorectal cancer patients. Although the taurine level in muscle was normal in tumor bearing mice, the tumor to muscle ratio was higher than the inflammation to muscle ratio. It suggests that higher accumulation in tumor can be distinguished from the surrounding inflammation which is accompanied with tumor.

Table 1. Biodistribution ratios at 1 h after the injection of [^{3}H]taurine

Ratio	Tumor (n=4)	Inflammation (n=5)	Tumor & Inflammation (n=5)
Tumor/Blood	8.1 ± 2.5[a]	ND	6.6 ± 1.7
Inflammation/Blood	ND[b]	6.4 ± 1.8	3.2 ± 0.9
Tumor/Muscle	2.6 ± 0.3	ND	2.4 ± 1.1
Inflammation/Muscle	ND	1.4 ± 0.3	1.4 ± 0.3

[a] Mean ± SD; [b] ND: not determined.

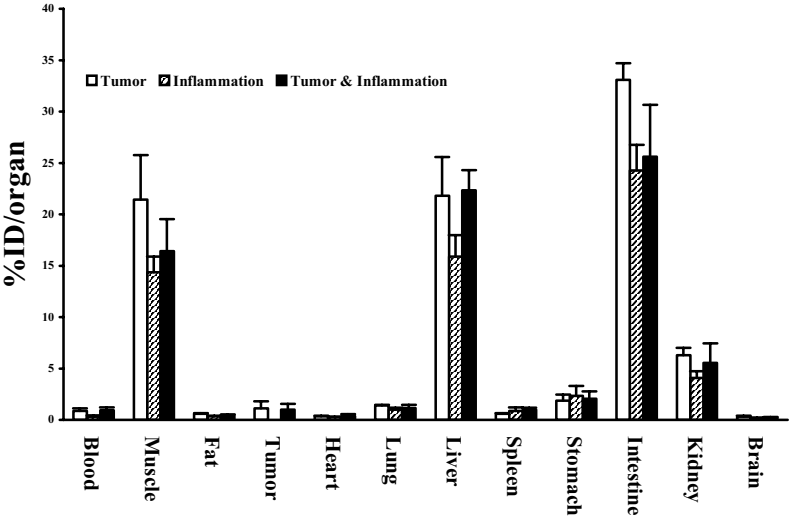

Figure 2. Organ distributions of [^{3}H]taurine in ICR mice having tumor and chronic inflammatory lesions at 1 h post injection. The data are expressed in percent of injected dose (% ID) per organ (n=5, inflammation group and inflammation plus tumor group; n=4, tumor group).

The high retention in the muscle, liver, intestine and kidney was observed (Fig. 2). In addition, the rapid clearance from the blood and negligible accumulation in the brain were observed (Figs. 1 and 2). This result is consistent with the autoradiography obtained from [^{35}S]taurine-injected mice (Shimono and Shimada, 1988). In conclusion, the results obtained from bio-distribution of taurine in tumor and inflammation suggest that the possibility of labeled taurine as a tumor tracing reagent.

4. ACKNOWLEDGMENTS

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Part 3.
Effects of Taurine Supplementation

EFFECTS OF GARLIC POWDER AND TAURINE SUPPLEMENTATION ON ABDOMINAL FAT, MUSCLE WEIGHT, AND BLOOD AMINO ACID PATTERN IN OVARIECTOMIZED RATS

Sun Hee Cheong, Chai Hyeock Yu, Mi-Ja Choi, and Kyung Ja Chang *

1. INTRODUCTION

Natural or surgically induced menopause is associated with increased levels of blood total cholesterol, triglyceride, LDL-cholesterol and risk for coronary heart disease (CHD) (Cuevas *et al*., 2003; Kishida *et al*., 2003). These changes are a consequence of reduction in the estrogen level (Kishida *et al*., 2003). Previous studies suggest that taurine and garlic have beneficial effects on cholesterol metabolism and atherosclerosis in postmenopausal women (Gandhi *et al*., 1992; Kishida *et al*., 2003; Tanamai *et al*., 2004). Taurine (2-aminoethanesulfonic acid) is a sulfur-containing β-amino acid that is the most abundant free amino acid in many mammalian tissues. It has many physiological functions including conjugation with bile acids, modulation of calcium levels, osmolarity maintenance, antioxidation, membrane protection, *etc.* (Redmond *et al*., 1998; Sethupathy *et al*., 2002). Garlic is one of the most commonly used herbal remedies and considered to have antihyperlipidemic as well as other cardioprotective properties in many human and animal studies (Peleg *et al*., 2003). It is also reported that plasma free amino acid levels may reflect the status of blood lipids and protein breakdown and utilization in obese subjects (Jeevanandam *et al*., 1991). However, the effects of taurine and garlic have been only little studied on the blood amino acid pattern in postmenopausal hyperlipidemic women. Therefore, this study was conducted in order to examine the effect of dietary taurine and garlic powder supplementation on abdominal fat, muscle weight and blood amino acid pattern in hyperlipidemic rats induced by ovariectomy.

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Taurine 6
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2. MATERIALS AND METHODS

2.1 Animals and Diet

Thirty-six female Sprague-Dawley rats weighing 200 g were supplied from Biolink (Seoul, Korea) and kept in shoe-box cages in a room with controlled temperature (23±2°C), humidity (55±5%), and light-dark-cycle (07:00-19:00). The rats were fed a commercial diet for one week. All rats were ovariectomized and then randomly assigned to four groups (high-fat diet group; HFD group, 2% freeze-dried garlic powder group; GP group, 1% taurine group; TR group, 2% freeze-dried garlic powder+1% taurine group; GP+TR group). The rats were fed experimental diets for 8 weeks (Table 1). Diets and water were provided *ad libitum*.

Table 1. Composition of experimental diet (g/100 g diet)

	High-fat group	Freeze-dried garlic powder (2%) group	Taurine (1%) group	Freeze-dried garlic powder (2%) + Taurine (1%) group
Casein	20	20	20	20
Corn starch	55	53	54	52
Corn oil	10	10	10	10
Beef tallow	5	5	5	5
A-Cellulose	5	5	5	5
Mineral mixture	4	4	4	4
Vitamin mixture	1	1	1	1
DL-Methionine	0.3	0.3	0.3	0.3
Freeze-dried garlic powder	0	2	0	2
Taurine	0	0	1	1

1) AIN mineral mixture (g/kg): calcium phosphate dibasic 500 g, sodium chloride 74 g, potassium citrate monohydrate 220 g, potassium sulfate 52 g, magnesium oxide 24 g, manganous carbonate (43-48% Mn) 3.5 g, ferric citrate (16-17% Fe) 6 g, zinc carbonate (70% ZnO) 1.6 g, cupric carbonate (53-55% Cu) 0.3 g, potassium iodate 0.01 g, sodium selenite 0.01 g, chromium potassium sulfate 0.55 g, and sucrose finely powdered 118 g.

2) AIN vitamin mixture (g/kg): thiamin hydrochloride 600 mg, riboflavin 600 mg, pyridoxine hydrochloride 700 mg, nicotinic acid 3 g, D-calcium pantothenate 1.6 g, folic acid 200 mg, D-biotin 20 mg, cyanocobalamin 1 mg, tetinyl palmitate pre-mix (250,000 IU/g) 1.6 g, DL-alpha-tocopherol acetate (250 IU/g) 20 g, cholecalciferol (400,000 IU/g) 250 mg, menaquinone 5 mg and sucrose finely powdered 972.9 g.

2.2. Blood Sampling and Analyses

The rats were sacrificed after 8 weeks of feeding experimental diets. The weights of liver, abdominal adipose tissue, and gastrocnemius and psoas muscles were measured. The size of abdominal adipose cells was examined by light microscopy. Blood samples were obtained through heart puncture and plasma was separated at 1400 rpm for 15 min. Plasma was stored at -70°C until analysis. The plasma free amino acid concentrations were measured using an automated amino acid analyzer based on ion exchanger chromatography (Biochrom 20, England).

2.3. Statistical Analysis

The statistical analysis was conducted using the SPSS 10.0 program. The means and standard deviations were calculated for all variables and analyzed by one-way ANOVA. The correlation between the weight of adipose tissue and concentrations of plasma free amino acids were analyzed using Pearson's correlation coefficient.

3. RESULTS AND DISCUSSION

The abdominal adipose tissue, liver, gastrocnemius and psoas muscle weights are shown in Table 2. The final body weights of all rats were not significantly different. The relative liver weight in GP+TR group was significantly lower than in the other groups ($p<0.05$). The weights of gastrocnemius and psoas muscle in the GP group were significantly higher than in the other groups ($p<0.05$). In a previous study, dietary taurine decreased the abdominal adipose tissue weight in hyperlipidemic rats (Gandhi *et al*., 1992). In this study, however, there were no significant differences in the weights of abdominal adipose tissue, although the weights of abdominal adipose tissue of the GP and/or) TR groups looked higher compared to those in the HFD group. These results suggest that short-term garlic and/or taurine supplementation dose not affect the weight of abdominal adipose tissue.

Table 2. Final body weight, weights of abdominal adipose tissue, liver, gastrocnemius and psoas muscles

Group	Final body weight (g)	Abdominal adipose tissue (g)	Relative liver (g)/ 100 g BW	Gastrocnemius muscle (g)	Psoas muscle (g)
HFD	273.3 ± 13.8 $^{N.S.}$	1.0 ± 0.3 $^{N.S.}$	7.7 ± 0.7 ^{b}	2.0 ± 0.2 ^{a}	1.8 ± 0.1 ^{a}
GP	287.5 ± 11.2	0.9 ± 0.2	8.1 ± 1.1 ^{b}	2.2 ± 0.2 ^{b}	2.0 ± 0.1 ^{b}
TR	265.6 ± 15.7	0.9 ± 0.1	7.7 ± 0.1 ^{b}	2.0 ± 0.1 ^{a}	1.7 ± 0.2 ^{a}
GP+TR	271.9 ± 17.3	0.8 ± 0.2	6.7 ± 0.0 ^{a}	1.9 ± 0.1 ^{a}	1.5 ± 0.1 ^{a}

Experimental rats were ovariectomized and then were randomly assigned to four groups (high-fat diet group; HFD group, 2% freeze-dried garlic powder group; GP group, 1% taurine group; TR group, 2% freeze-dried garlic powder+1% taurine group; GP+TR group). Values are means ± SD. Means in each column not sharing a common superscript letter are significantly different at $p<0.05$ by Duncan's multiple range test.

The sizes of abdominal adipose cells are shown in Figure 1. The sizes of abdominal adipose cells in the GP and GP+TR groups were different from those in the HFD group. These results suggest that the sizes of abdominal adipose cell may reflect the status of obesity. In a previous study it was reported that taurine supplementation has an important role in cardiovascular disease prevention in overweight or obese subjects (Sethupathy *et al*., 2002).

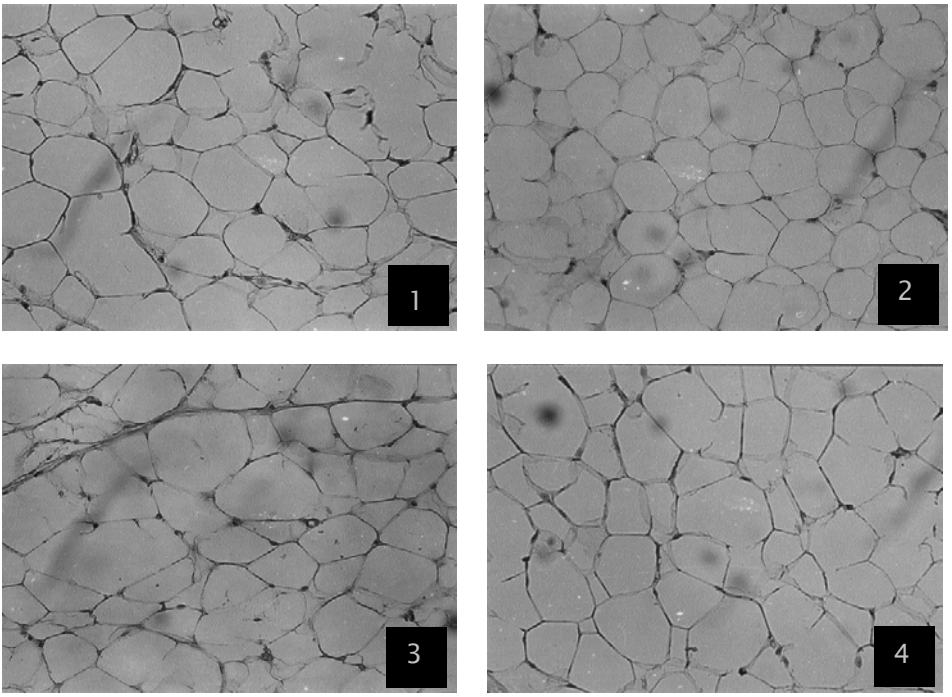

Figure 1. Size of abdominal adipose cell examined by light microscopy. (Bar = 100 μm). Experimental rats were ovariectomized and then were randomly assigned to four groups (1: high-fat diet group; HFD group, 2: 2% freeze-dried garlic powder group; GP group, 3: 1% taurine group; TR group, 4: 2% freeze-dried garlic powder+1% taurine group; GP+TR group).

The plasma free amino acid concentrations are shown in Table 3. The plasma taurine concentration in the HFD and GP groups is significantly lower than in the TR and GP+TR groups ($p<0.05$). These results show that the plasma taurine level reflects dietary taurine. The plasma proline, valine, ammonium chloride, histidine and arginine concentrations in the GP+TR group are significantly higher than in the other groups ($p<0.05$). On the other hand, the plasma serine and tyrosine concentrations in the GP group are the highest when compared to those in the other groups ($p<0.05$). In a previous study conducted with obese traumatized man, decreases were observed in the glycine, tryptophan, threonine, histidine, taurine, citrulline, and cystine levels (Jeevanandam *et al*., 1991).

Table 4 shows the correlation coefficient between the weight of abdominal adipose tissue and the concentrations of plasma free amino acids. The weight of abdominal fat was positively correlated with the plasma cystine ($p<0.01$), cystathionine ($p<0.01$), hydroxylysine ($p<0.01$) and arginine concentrations ($p<0.05$). Several human and animal studies have indicated that the elevated plasma homocysteine and cysteine level is a strong risk factor for coronary heart disease (Jacob *et al*., 1999; Dzielinska *et al*., 2000; Bozkurt *et al*., 2003). Also a 5-μmol/l increment in the total homocysteine level may be associated with a twofold increase of risk for the disease (Dzielinska *et al*., 2000). These results suggest that plasma cysteine is strongly related with the adipose tissue weight being the risk factor for atherosclerosis in postmenopausal women.

Table 3. Concentrations of plasma free amino acids (μmol/l)

Group	HFD	GP	TR	GP+TR
Taurine	79.2 ± 10.2[a]	83.6 ± 16.5[a]	279.5 ± 17.5[b]	363.2 ± 20.1[b]
Proline	609.2 ± 11.4[a]	779.9 ± 36.1[a]	617.7 ± 10.5[a]	1170.6 ± 25.7[b]
Cystine	0.9 ± 0.1[N.S.]	0.9 ± 0.0	1.0 ± 0.2	1.1 ± 0.1
Cystathionine	25.1 ± 2.1[N.S.]	23.9 ± 1.8	24.0 ± 1.4	23.0 ± 1.1
Hydroxylysine	1.9 ± 0.1[N.S.]	1.8 ± 0.0	1.7 ± 0.0	1.7 ± 0.2
Serine	197.3 ± 39.0[a]	255.7 ± 40.2[b]	168.3 ± 16.6[a]	146.3 ± 12.6[a]
Asparagine	63.7 ± 17.2[b]	72.8 ± 14.5[b]	28.9 ± 4.2[a]	81.2 ± 15.3[b]
Glutamic acid	227.4 ± 14.8[b]	202.1 ± 11.4[b]	142.6 ± 13.3[a]	244.3 ± 13.2[b]
Glycine	142.3 ± 14.2[a]	312.0 ± 20.4[b]	227.1 ± 33.4[ab]	326.4 ± 18.1[bc]
Citrulline	41.8 ± 9.4[b]	22.7 ± 5.7[a]	44.4 ± 3.4[b]	24.4 ± 4.5[a]
Alanine	330.6 ± 15.1 [N.S.]	292.1 ± 20.0	418.5 ± 17.6	466.8 ± 18.4
Valine	110.9 ± 22.4[ab]	75.7 ± 6.9[a]	124.7 ± 13.5[ab]	135.1 ± 14.2[b]
Methionine	36.9 ± 7.2 [N.S.]	53.9 ± 4.9	46.3 ± 7.2	41.5 ± 7.6
Isoleucine	55.2 ± 3.3 [N.S.]	84.3 ± 3.1	58.9 ± 12.0	71.9 ± 9.7
Leucine	103.6 ± 12.1 [N.S.]	93.3 ± 5.2	112.5 ± 4.2	135.9 ± 7.8
Tyrosine	75.6 ± 6.8 [ab]	147.5 ± 12.2[b]	47.1 ± 7.2[a]	116.3 ± 6.0 [ab]
Phenylalanine	37.9 ± 1.3 [N.S]	43.9 ± 3.2	45.1 ± 1.7	52.8 ± 6.0
γ-Amino-n-butyric acid	29.5 ± 2.1[b]	11.5 ± 6.7[a]	11.8 ± 1.7[a]	21.4 ± 6.1[ab]
Ammonium chloride	264.5 ± 16.4[a]	324.5 ± 15.1[ab]	344.1 ± 13.3[ab]	403.3 ± 19.2[b]
Ornithine	80.3 ± 5.6[b]	55.8 ± 3.3[a]	93.3 ± 4.1[b]	97.1 ± 7.0[b]
Lysine	304.7 ± 5.1[N.S.]	359.3 ± 4.4	308.9 ± 6.7	323.0 ± 7.0
Histidine	37.8 ± 1.2[a]	43.7 ± 3.4[ab]	41.7 ± 7.9[ab]	49.7 ± 1.9[b]
Arginine	114.1 ± 7.6[a]	127.8 ± 5.7[ab]	117.1 ± 7.4[a]	149.0 ± 8.2[b]

Experimental rats were ovariectomized and then randomly assigned to four groups (high-fat diet group; HFD group, 2% freeze-dried garlic powder group; GP group, 1% taurine group; TR group, 2% and freeze-dried garlic powder+1% taurine group; GP+TR group). Values are means ± SD. The means in each column not sharing a common superscript letter are significantly different at $p<0.05$ by Duncan's multiple range test.

Table 4. Correlation coefficient between adipose tissue weight and plasma free amino acid concentrations

	Cysteine	Cystathionine	Hydroxylysine	Arginine
Adipose tissue	0.589[**]	0.574[**]	0.573[**]	0.430[*]

Correlation coefficients between adipose tissue and plasma free amino acid concentrations are significant at $p<0.05$ and at $p<0.01$ by Pearson correlation.

The above results may suggest that taurine and garlic powder reduce the sizes of abdominal adipose cells and that the blood amino acid pattern including cystine, cystathionine, hydroxylysine and arginine is correlated with the abdominal fat in postmenopausal model rats. However, because the plasma homocysteine level was not measured, further studies are needed using the these preliminary data.

4. ACKNOWLEDGMENTS

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EFFECTS OF GARLIC POWDER AND SOY PROTEIN SUPPLEMENTATION ON BLOOD LIPID PROFILES AND AMINO ACID CONCENTRATIONS IN POSTMENOPAUSAL HYPERLIPIDEMIC MODEL RATS

Sun Hee Cheong, Mi-Ja Choi, and Kyung Ja Chang *

1. INTRODUCTION

High serum lipid levels such as total cholesterol, triglycerides and LDL-cholesterol have long been known as a risk factor for cardiovascular disease in many experimental animals and humans (Almario *et al*., 2001; Rowley *et al*., 2001). The major cause for hyperlipidemia has been associated with increasing age, sexual difference, obesity, inappropriate food intake, increased dietary fat intake, high blood pressure, high blood glucose, stress, smoking, and changes in hormonal and environmental factors (Kim *et al*., 2000; Rowley *et al*., 2001). Especially, it has been reported that the hyperlipidemia risk factor of postmenopausal women has increased due to changes in blood lipid metabolism and increased insulin resistance due to reduced synthesis of estrogens in the ovary (Kim *et al*., 2000; Seed, 2002). Numerous studies suggest that garlic and soy protein have antiatherosclerotic effects (Ali *et al*., 2000; Peleg *et al*., 2003; Satitvipawee *et al*., 2003). The use of aqueous garlic extract is known to be effective in reducing thromboxane formation by platelets both *in vivo* and *in vitro* (Satitvipawee *et al*., 2003). The antiatherogenic mechanisms of isoflavones are not known, but many studies suggest that soy protein may reduce the increased formation of atherosclerotic lesions in women after menopause (Isaacsohn *et al*., 1998). Recently, it has been also reported soy protein has beneficial effects on lipid changes, vascular reactivity and markers of inflammation in postmenopausal women (Lissin *et al*., 2004; Zhan and Ho, 2005). It is thought that the plasma lipid and free amino acid concentrations in postmenopausal women may be influenced by dietary garlic powder and soy protein supplementation. Numerous studies

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have shown that soy protein or garlic powder lowers blood lipids. However, there are only few studies on the effects of garlic powder and soy proteins administered simultaneously to postmenopausal hyperlipidemic rats. Therefore, the present study was conducted in order to examine the effects of dietary garlic powder and soy protein supplementation on the blood lipid profiles and free amino acid pattern in postmenopausal hyperlipidemic model rats.

2. MATERIALS AND METHODS

2.1. Animals and Diet

Thirty-six female Sprague-Dawley rats weighing 200 g were supplied from Animal Care Facility of Seoul National University (Seoul, Korea) and were kept in polycarbonate cages in a room with controlled temperature (23 ± 2°C), humidity (55 ± 5%) and light-dark-cycle (07:00-19:00). The rats were fed a commercial diet for one week. All rats were ovariectomized and then randomly assigned to four groups (casein high-fat group; CHF group, casein high-fat +2% freeze-dried garlic powder group CHF+GP group, soy protein high-fat group; SHF group, and soy protein high-fat +2% freeze-dried garlic powder group; SHF+GP group). The rats were fed experimental diets for 8 weeks (Table 1).

Table 1. Composition of experimental diet (g/100 g diet)

	Casein high-fat group	Casein high-fat +2% freeze-dried garlic powder group	Soy protein high-fat group	Soy protein high-fat +2% freeze-dried garlic powder group
Casein	20	20	0	0
Soy protein	0	0	20	20
Corn starch	55	53	55	53
Corn oil	10	10	10	10
Beef tallow	5	5	5	5
A-Cellulose	5	5	5	5
Mineral mixture[1]	4	4	4	4
Vitamin mixture[2]	1	1	1	1
DL-Methionine	0.3	0.3	0.3	0.3
Freeze-dried garlic powder	0	2	0	2

(1) AIN mineral mixture (g/kg): calcium phosphate dibasic 500 g, sodium chloride 74 g, potassium citrate monohydrate 220 g, potassium sulfate 52 g, magnesium oxide 24 g, manganous carbonate (43-48% Mn) 3.5 g, Ferric citrate (16-17% Fe) 6 g, zinc carbonate (70% ZnO) 1.6 g, cupric carbonate (53-55% Cu) 0.3 g, potassium iodate 0.01 g, sodium selenite 0.01 g, chromium potassium sulfate 0.55 g, and sucrose, finely powdered, 118 g.

(2) AIN vitamin mixture (g/kg): thiamin hydrochloride 600 mg, riboflavin 600 mg, pyridoxine hydrochloride 700 mg, nicotinic acid 3 g, D-calcium pantothenate 1.6 g, folic acid 200 mg, D-biotin 20 mg, cyanocobalamin 1 mg, retinyl palmitate pre-mix (250,000 IU/g) 1.6 g, DL-alpha-tocopherol acetate (250 IU/g) 20 g, cholecalciferol (400,000 IU/g) 250 mg, menaquinone 5 mg, and sucrose, finely powdered 972.9 g.

2.2. Blood Sampling and Analysis

The rats were sacrificed after 8 weeks of feeding experimental diets. The weights of liver, abdominal adipose tissue, and gastrocnemius and psoas muscles were measured. Blood samples were obtained through heart puncture and serum was separated at 3000 rpm for 20 min. Plasma was separated by centrifugation at 1400 rpm for 15 min. Serum and plasma were stored at -70°C until analyses. The serum triglyceride, total cholesterol and HDL-cholesterol concentrations were analyzed by enzymatic procedures using commercial kits with UV/Vis spectrophotometer at 546 nm, 500 nm and 500 nm, respectively (Youngdong Pharmaceutical Co., Korea). Serum LDL-cholesterol was calculated with the formula: LDL-cholesterol = total cholesterol – (HDL-cholesterol + triglycerides/5) (Friedewald *et al.*, 1972). Also the HDL-cholesterol/total cholesterol and LDL-cholesterol/HDL-cholesterol ratios were calculated. The plasma free amino acid concentrations were measured using an automated amino acid analyzer based on ion exchanger chromatography (Biochrom 20, England).

2.3. Statistical Analysis

The statistical analysis was conducted using the SPSS 10.0 program. The means and standard deviation were calculated for all variables and analyzed by one-way ANOVA. The correlation among the weights of adipose tissue, liver, muscle, and blood lipid and plasma free amino acid levels was evaluated with Pearson's correlation coefficient.

3. RESULTS AND DISCUSSION

The weights of abdominal adipose tissue, liver, and gastrocnemius and psoas muscles are shown in Table 2. The final body weights of all rats were not significantly different among groups. The weight of abdominal adipose tissue in the SHF+GP group was significantly lower than in the other groups ($p<0.05$). The weight of gastrocnemius muscle in the SHF group was significantly higher than in the other groups ($p<0.05$) and the weight of psoas muscle in the CHF+GP group significantly higher than in the other groups ($p<0.05$). These results suggest that soy protein added to garlic powder increases the gastrocnemius muscle weight but decreases the abdominal adipose tissue weight.

Table 2. Final body weight, abdominal adipose tissue, liver, and gastricnemus and psoas muscle weights

Group	Final body weight (g)	Abdominal adipose tissue (g)	Relative liver (g)/100 g BW	Gatrocnemius muscle (g)	Psoas muscle (g)
CHF	273.3 ± 13.8 $^{N.S.}$	1.0 ± 0.3 ^{b}	7.7 ± 0.7 $^{N.S.}$	2.0 ± 0.2 ^{a}	1.8 ± 0.1 ^{a}
CHF+GP	287.5 ± 11.2	0.9 ± 0.2 ^{b}	8.1 ± 1.1	2.2 ± 0.2 ^{a}	2.0 ± 0.1 ^{b}
SHF	265.0 ± 12.1	0.8 ± 0.1 ^{b}	7.8 ± 0.3	2.6 ± 0.1^{b}	1.6 ± 0.1 ^{a}
SHF+GP	258.3 ± 14.2	0.6 ± 0.1 ^{a}	7.5 ± 0.2	2.0 ± 0.2^{a}	1.7 ± 0.2 ^{a}

Experimental rats were ovariectomized and then randomly assigned to four groups (casein high-fat group; CHF group, casein high-fat +2% freeze-dried garlic powder group CHF+GP group, soy protein high-fat group; SHF group, and soy protein high-fat +2% freeze-dried garlic powder group; SHF+GP group). Values are means ± SD. The means in each column not sharing a common superscript letter are significantly different at $p<0.05$ by Duncan's multiple range test.

The serum lipid profiles are shown in Table 3. The serum total cholesterol level in the SHF and SHF+GP groups was significantly lower than in the CHF group ($p<0.05$). The serum triglyceride level in the CHF group was higher than in the other groups ($p<0.05$). HDL-cholesterol and the ratio of HDL-cholesterol to total cholesterol were highest in the SHF+GP group. There was also a significant difference in HDL-cholesterol and the ratio of HDL-cholesterol to total cholesterol between the CHF and CHF+GP groups. On the other hand, the LDL-cholesterol levels and the ratio of LDL-cholesterol to HDL-cholesterol in the CHF group were significantly higher than in the other groups ($p<0.05$). These results showed that garlic powder and soy protein when compared with casein have an effect in increasing the HDL-cholesterol level and synergistic effects obtain between soy protein and garlic powder on the serum total cholesterol level. In previous studies, garlic powder has been beneficial in reducing blood cholesterol, triglyceride levels in hypercholesterolemic rats (Ali *et al*., 2000). However, in a previous study conducted with hypercholesterolemic subjects, treatment with an enteric garlic-coated Thai garlic extract for 6 months did not produce any significant changes in blood lipid levels (Tanamai *et al*., 2004). It is considered that the ratio of LDL-cholesterol to HDL-cholesterol may be a strong predictor of cardiac events (Hermansen *et al*., 2003). These authors reported that consumption of soy products significantly reduced the ratio LDL-cholesterol to HDL-cholesterol up to 27%. In a previous study, favorable effects have been reported of 30-50 g soy protein compared with casein on the total cholesterol, LDL-cholesterol and total homocysteine concentrations in blood plasma in hyperlipidemic subjects (Tonstad *et al*., 1991), which findings are similar to the results of the present study. It has been also reported that consumption of soy protein decreased plasma total cholesterol by 2.2% and LDL-cholesterol by 3.5%, and increased HDL-cholesterol by 4.2% when compared with milk protein in postmenopausal women (Jeevanandam *et al*., 2002).

Table 3. Serum lipid profiles of rats fed experimental diet for 8 weeks

Group	TC (mg/dl)	Triglyceride (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)	HDL-C/ TC (ratio)	LDL-C/ HDL-C (ratio)
CHF	157.6 ± 21.2^{b}	142.3 ± 18.0^{b}	28.8 ± 8.6^{a}	123.4 ± 3.6^{c}	0.2 ± 0.1^{a}	2.0 ± 0.2^{b}
CHF+GP	106.8 ± 20.1^{ab}	107.6 ± 11.2^{a}	55.6 ± 7.1^{b}	74.1 ± 2.2^{b}	0.5 ± 0.1^{b}	1.4 ± 0.1^{a}
SHF	93.0 ± 8.2^{a}	97.2 ± 6.5^{a}	55.9 ± 9.7^{b}	62.4 ± 3.2^{a}	0.6 ± 0.1^{b}	1.2 ± 0.1^{a}
SHF+GP	97.5 ± 8.6^{a}	109.3 ± 5.4^{a}	66.9 ± 7.0^{c}	62.2 ± 7.4^{a}	0.7 ± 0.1^{b}	1.0 ± 0.1^{a}

Experimental rats were ovariectomized and then randomly assigned to four groups (casein high-fat group; CHF group, casein high-fat +2% freeze-dried garlic powder group CHF+GP group, soy protein high-fat group; SHF group, and soy protein high-fat +2% freeze-dried garlic powder group; SHF+GP group). Values are means ± SD. The means in each column not sharing a common superscript letter are significantly different at $p<0.05$ by Duncan's multiple range test.

The plasma free amino acid concentrations of rats are shown in Table 4. The plasma taurine concentrations in the SHF and SHF+GP groups were significantly higher than in the CHF group ($p<0.05$). The plasma serine and tyrosine concentrations in the CHF+GP group were significantly higher than in the other groups ($p<0.05$). On the other hand, the plasma proline, asparagine, leucine, phenylalanine, ammonium chloride, ornithine and histidine concentrations in the SHF group were significantly higher than in the other

groups ($p<0.05$). The plasma glycine, alanine and citrulline concentrations in the SHF+GP group were significantly higher than in the other groups ($p<0.05$). Recently, it is reported that the plasma levels of circulating free amino acids reflect the net status of protein breakdown and utilization, and lipid metabolism. Jeevanadam *et al.* (2002) reported that obese subjects showed an increase in the valine, leucine, isoleucine and glutamic acid levels and a decrease in the glycine, tryptophan, threonine, histidine, taurine, citrulline and cystine levels when compared with lean subjects.

Table 4. Concentrations of plasma free amino acids (μmol/l)

Group	CHF	CHF+GP	SHF	SHF+GP
Taurine	79.2 ± 10.2^{a}	83.6 ± 16.5^{ab}	305.1 ± 17.3^{c}	284.8 ± 5.6^{c}
Proline	609.2 ± 11.4^{a}	779.9 ± 36.1^{a}	1016.1 ± 21.5^{c}	954.6 ± 15.3^{b}
Cystine	$0.9 \pm 0.1^{N.S.}$	0.9 ± 0.2	1.1 ± 0.0	1.0 ± 0.0
Cystathionine	$25.1 \pm 2.1^{N.S.}$	23.9 ± 1.8	23.4 ± 1.2	24.1 ± 1.5
Hydroxylysine	$1.9 \pm 0.1^{N.S.}$	1.8 ± 0.0	1.9 ± 0.2	1.7 ± 0.2
Serine	197.3 ± 39.0^{b}	255.7 ± 40.2^{c}	57.7 ± 8.7^{a}	125.1 ± 10.4^{b}
Asparagine	63.7 ± 17.2^{a}	72.8 ± 14.5^{a}	106.0 ± 13.2^{b}	71.6 ± 4.1^{a}
Glutamic acid	$227.4 \pm 14.8^{N.S.}$	202.1 ± 11.4	215.7 ± 10.9	262.6 ± 13.9
Glycine	142.3 ± 14.2^{a}	312.0 ± 20.4^{bc}	216.4 ± 16.8^{b}	318.4 ± 4.2^{bc}
Citrulline	41.8 ± 9.4^{b}	22.7 ± 5.7^{a}	15.8 ± 5.9^{a}	43.6 ± 8.0^{ab}
Alanine	330.6 ± 15.1^{a}	292.1 ± 20.0^{a}	488.7 ± 17.9^{b}	479.9 ± 9.2^{b}
Valine	110.9 ± 22.4^{b}	75.7 ± 6.9^{a}	149.6 ± 16.9^{b}	109.5 ± 15.4^{b}
Methionine	$36.9 \pm 7.2^{N.S.}$	53.9 ± 4.9	49.7 ± 2.5	43.2 ± 1.7
Isoleucine	55.2 ± 3.3^{a}	84.3 ± 3.1^{b}	60.9 ± 3.1^{ab}	62.5 ± 9.9^{ab}
Leucine	103.6 ± 12.1^{ab}	93.3 ± 5.2^{a}	139.2 ± 6.2^{b}	113.3 ± 5.7^{ab}
Tyrosine	75.6 ± 6.8^{ab}	147.5 ± 12.2^{b}	92.4 ± 12.1^{ab}	35.0 ± 2.0^{a}
Phenylalanine	37.9 ± 1.3^{a}	43.9 ± 3.2^{a}	54.7 ± 8.6^{b}	44.9 ± 6.0^{a}
γ-Amino-n-butyric acid	29.5 ± 2.1^{b}	11.5 ± 6.7^{a}	19.2 ± 1.0^{ab}	14.1 ± 1.5^{a}
Ammonium chloride	264.5 ± 16.4^{a}	324.5 ± 15.1^{ab}	599.6 ± 15.8^{b}	397.4 ± 9.5^{ab}
Ornithine	80.3 ± 5.6^{ab}	55.8 ± 3.3^{a}	131.7 ± 7.3^{b}	106.8 ± 14.5^{ab}
Lysine	$304.7 \pm 5.1^{N.S.}$	359.3 ± 4.4	378.1 ± 5.7	307.0 ± 5.2
Histidine	37.8 ± 1.2^{a}	43.7 ± 3.4^{b}	56.2 ± 6.5^{c}	43.9 ± 7.2^{b}
Arginine	$114.1 \pm 7.6^{N.S.}$	127.8 ± 5.7	137.0 ± 6.1	132.0 ± 9.6

Experimental rats were ovariectomized and then randomly assigned to four groups (casein high-fat group; CHF group, soy protein high-fat group; SHF group, casein high-fat +2% freeze-dried garlic powder group CHF+GP group, and soy protein high-fat +2% freeze-dried garlic powder group; SHF+GP group). Values are means ± SD. The means in each column not sharing a common superscript letter are significantly different at $p<0.05$ by Duncan's multiple range test.

The correlation coefficients among the adipose tissue weight, blood lipid levels and plasma free amino acid concentrations are shown in Table 5. The weight of abdominal fat was positively correlated with the plasma cystine and hydroxylysine concentrations, but negatively correlated with the plasma glutamic acid and leucine concentrations. Blood total cholesterol, triglycerides, LDL-cholesterol concentrations and the ratio of LDL-

cholesterol to HDL-cholesterol were positively correlated with the plasma threonine, aminoadipic acid and citrulline concentrations, but negatively with the plasma taurine, urea, proline, phenylalanine, lysine and histidine concentrations. In a previous study, it was reported that blood total cholesterol was inversely related to phenylalanine, cystathionine, methionine and glycine (Chiarla *et al*., 2004). Many previous studies indicate that the plasma cysteine and homocysteine levels are positively correlated with blood total cholesterol, LDL-cholesterol and the risk factor of cardiovascular disease (Jacob *et al*., 1999; Dzielinska *et al*., 2000; Bozkurt *et al*., 2003; Racek *et al*., 2005). On the other hand, plasma HDL-cholesterol and the ratio of HDL-cholesterol to total cholesterol were positively correlated with taurine, proline, phenylalanine, methyl-l-histidine and histidine but negatively with threonine and citrulline.

Table 5. Correlation coefficients among adipose tissue weight, blood lipid levels and plasma free amino acid concentrations

	Adipose tissue	TC	TG	HDL-C	LDL-C	HDL-C/TC	LDL-C/HDL-C
Serine	0.302	-0.375	-0.577**	-0.083	-0.291	0.026	-0.231
Taurine	-0.122	-0.686**	-0.598**	0.478*	-0.659**	0.617**	-0.609**
Proline	-0.277	-0.513*	-0.684**	0.603**	-0.483*	0.550*	-0.596**
Threonine	0.076	0.461*	0.477*	-0.385	0.439*	-0.461*	0.395
Asparagine	-0.175	-0.091	-0.150	0.339	-0.104	0.255	-0.165
Aminoadipic acid	-0.151	0.478*	0.121	-0.094	0.479*	-0.368	0.164
Alanine	-0.342	0.419	0.140	-0.206	0.427*	-0.243	0.312
Citrulline	0.007	0.454*	0.576**	-0.440*	0.423	-0.355	0.642**
Cystine	0.614*	-0.115	-0.102	-0.073	-0.096	-0.047	-0.098
Leucine	-0.711**	-0.161	-0.200	0.326	-0.166	0.318	-0.194
Phenylalanine	-0.295	-0.480*	-0.479*	0.498*	-0.468*	0.580**	-0.421
Hydroxylysine	0.614*	-0.115	-0.102	-0.073	-0.096	-0.047	-0.094
Ornithine	-0.168	-0.063	-0.083	0.225	-0.074	0.223	-0.052
Lysine	-0.320	-0.469*	-0.594**	0.244	-0.416	0.243	-0.504**
Histidine	-0.170	-0.547*	-0.476*	0.468*	-0.534*	0.589**	-0.468*
Methyl-l-histidine	-0.131	-0.106	-0.353	0.602**	-0.116	0.368	-0.331

Correlation coefficients among serum lipid levels are significant at $p<0.05$ and at $p<0.01$ by Pearson correlation.

The above results may suggest a blood triglyceride, total cholesterol, LDL-cholesterol lowering effect of garlic powder and soy protein and some correlation of abdominal adipose tissue and blood lipid profiles with the blood amino acid pattern in postmenopausal hyperlipidemic model rats. However, because this study was conducted with experimental animals, further studies should be conducted to show the effects to improve blood lipid profiles without adverse effects with long-term supplementation in elderly women.

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THE EFFECT OF DIETARY TAURINE SUPPLEMENTATION ON PLASMA AND LIVER LIPID CONCENTRATIONS AND FREE AMINO ACID CONCENTRATIONS IN RATS FED A HIGH-CHOLESTEROL DIET

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1. ABSTRACT

The purpose of this study was to investigate the effect of dietary taurine supplementation on plasma and liver lipid concentrations, and free amino acid concentrations in rats fed a high-cholesterol diet. Twenty male rats (body weight 151 ± 1.9 g) were randomly divided into two groups. The rats in the control group were fed on 1.5% cholesterol diet (control) and those in the experimental group were fed with 1.5% cholesterol and 1.5% taurine diet (TSD). All rats were fed with the experimental diets and deionized water *ad libitum* for 5 weeks. The plasma glucose and lipid concentrations were measured using commercial kits with enzymatic methods and liver lipid concentrations with the Folch method. The concentrations of free amino acids in plasma were determined with an automated amino acid analyzer based on ion-exchange chromatography. There were no significant differences in the body weight gain, food intake and food efficiency ratio between the control and experimental groups. The rats fed TSD had significantly lower liver weight and liver weight/body weight ratio than those fed control diet. The plasma concentrations of total cholesterol, glucose and LDL-cholesterol were significantly reduced in the rats fed TSD compared to those fed control diet. The rats fed TSD showed significantly decreased liver levels of cholesterol and triglyceride. The HDL-cholesterol level was higher in the rats fed TSD than those fed control diet. The plasma taurine concentrations were not significantly different between two groups. They exhibited significant negative correlation with the plasma total cholesterol and liver triglyceride concentrations. These results suggest the possible role of taurine as a hypocholesterolemic agent in the rats fed a high cholesterol diet. Taurine

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supplementation did not cause any characteristic changes in the plasma aminogram pattern, body weight gain, and food intake.

2. INTRODUCTION

Numerous studies have been done on the effect of taurine on cholesterol metabolism (Petty *et al*., 1990, Yan *et al*., 1993, Murakami *et al*., 2002, Kishida *et al*., 2003) in various species, including rats, guinea pigs, rabbits, and cats. Almost all of the experiments have been done on animals with hypercholesterolemia induced by feeding a high-cholesterol diet. Recently, Nanami *et al*. (1996) also reported a hypocholesterolemic action of taurine in rats fed a high-cholesterol diet, but the mechanism of its action is unclear. Furthermore, Park *et al*. (1998) reported a hypocholesterolemic action of taurine in rats fed a high-cholesterol diet. However, addition of taurine to the diet did not reduce the serum level of cholesterol in rats (Mochizuki *et al*., 1998). Therefore, more studies are needed to figure out the beneficial effects of taurine on cholesterol metabolism. Moreover, none of the above-mentioned studies was performed predominantly in view of the plasma taurine concentration. With the increasing use of amino acids as dietary supplements, it is of importance to recognize those amino acids, which may prove to be potentially toxic and the conditions under which such toxicities would most likely be encountered. Taurine is thought to be quite safe and there is little concern about the side effects of excessive intake of taurine (Furukawa *et al*., 1991). In particular, little is known about the relationship between changes in plasma taurine and other amino acid levels.

There are a number of repots indicating modifications of taurine concentrations in plasma in specific conditions. Due to the lack of information concerning taurine, the aim of the present work was to determine the concentration of taurine and other amino acids in plasma in hyperlipidemic rats after supplementation with taurine. Therefore, we studied the possible relation between the plasma taurine concentration and plasma lipids.

3. MATERIALS AND METHODS

Male Sprague-Dawley rats weighing about 150 g (Seoul) were fed an AIN-76 standard rat diet. The rats were randomly assigned to 2 groups of 10 rats in each: those with a taurine-supplemented diet (taurine-supplemented group, TSD) and those without supplementation (unsupplemented group, control). The compositions of the experimental diets are shown in Table 1. All animals were fed the high-cholesterol diet. The rats were individually housed in stainless steel cages in a room with controlled temperature (23°C) and humidity (55%) and were given free access to the experimental diets and water. The rats were maintained in a 12-h light (07:00–19:00 h) and dark cycle.

Plasma was separated from blood by centrifugation (1600 x g, 15 min, 4^{o}C). Plasma lipids (total cholesterol, HDL-cholesterol and triglycerides) were determined by using commercial kits (Wako Pure Chemical, Osaka). The LDL-cholesterol concentrations were estimated with the equation of Friedewald *et al*. (1972). About 2 g of liver were homogenized, and lipids extracted with chloroform: methanol mixture (2:1 v/v), as described by Folch *et al*. (1957). The concentration of liver cholesterol in the lipid extracts was measured enzymatically by using a kit (Wako Pure Chemical, Osaka).

Table 1. Composition of experimental diets (g/100 g diet)

Ingredient	Dietary group	
	Control [1]	TSD [2]
Casein [3]	18	18
Corn starch [4]	63.5	62.0
Corn oil [5]	10	10
Cellulose [6]	2	2
Mineral mixture [7]	4	4
Vitamin mixture [8]	1	1
Cholesterol [9]	1.5	1.5
Taurine [10]	-	1.5

(1) Control: high cholesterol control diet. (2) TSD: high-cholesterol taurine-supplemented diet. (3) Casein, Maeil dairy industry Co., Ltd. 480 Gagok-Ri, Jinwi-Myun, Pyungtaek-City, Kyunggi-Do. (4) Corn starch, Doosan Co. 234-17 Maam-Ri, Bubal-Eup, Ichon-City, Kyunggi-Do. (5) Corn-oil, Shindong-bang oil Co. 4-2 Yangpyung-Dong, Youngdongpo-Gu Seoul: KSH 2102. (6) Cellulose, supplied by Sigma Chemical company No. C8002. (7) Mineral mixture, supplied by US Corning Laboratory Services Company, Teklad test diets, Madison, Wisconsin, Biological Test Material No.170915. (8) Vitamin mixture, supplied by US Corning Laboratory Services Company, Teklad Test Diets, Madison, Wisconsin, Biological Test Material No.40077. (9) Cholesterol, supplied by Sigma Chemical Company No. 2044. (10) Taurine, Dong-A Pharm. Co. Ltd. 434-4 Moknae-dong, Ansan-City, Kyunggi-Do.

Plasma was deproteinized by using sulfosalicylic acid, and taurine concentrations in plasma were measured with an automatic amino acid analyzer based on ion-exchange chromatography (Biochrom 20, Pharmacia Biotech, Cambridge).

The SAS statistical package (version 8.12; SAS Institute Inc, Cary, NC) was used for the analysis. To assess the mean difference for continuous variables between the control and experimental groups, Student's t-test for independent group was used. Pearson correlation coefficients were calculated to describe associations between the taurine concentrations and blood lipids. The results are expressed as means ± SD. Values were reported as significant when P values < 0.05.

4. RESULTS AND DISCUSSION

4.1. Weight Gain and FER

Table 2 shows the weight at beginning, weight at sacrifice, weight gain, food intake and food efficiency ratio (FER) of rats fed with the experimental diets. The body weight gain and food intake of rats fed the experimental diet (taurine 15 g/kg diet) did not differ from those of rats fed the control diet. This finding is in agreement with Sugiyama and co-workers (1989) who reported that taurine supplementation had no influence on the weight gain and food intake of the animals.

4.2. Liver Weight

The weight of liver was significantly lower in the rats fed taurine-supplemented diet than in the rats fed control diet (10.93 ± 0.95 g *vs.* 15.20 ± 0.86 g) (Table 3). This finding is in agreement with other studies. Yokogoshi and co-workers (1999) reported that taurine

supplementation significantly decreased (37%) the liver weight when the rats fed a high-cholesterol diet.

Table 2. Weight, food intake and FER of rats fed control and TSD diets during experimental period

Variables	Control	TSD	Significance
Weight at beginning (g)	150.1 ± 1.7 (1)	150.4 ± 1.9	NS (2)
Weight at sacrifice (g)	329.5 ± 13.1	323.9 ± 12.9	NS
Weight gain (g)	179.9 ± 8.6	172.4 ± 7.6	NS
Food intake (g/day)	21.91 ± 0.55	21.97 ± 1.29	NS
FER (3)	0.26 ± 0.07	0.23 ± 0.01	NS

(1) Values are means ± SD. (2) NS: Not significantly different at $p<0.05$ by Student's t-test. (3) FER: Food efficiency ratio.

4.3. Blood Glucose and Lipids

The effects of taurine supplementation on blood glucose and lipids in the rats with the high-cholesterol diet are shown in Table 4. The blood glucose concentration was significantly lower in the rats fed the taurine-supplemented diet than in the rats fed the control diet (96.97 ± 8.97 mg/dl *vs.* 120.55 ± 14.50 mg/dl). Indeed, taurine has been shown to reduce blood glucose levels acutely in diabetic rats (Nakaya *et al*., 2000).

The serum concentrations of total cholesterol (82 ± 5.15 mg/dl *vs.* 107.21 ± 15.76 mg/dl) and LDL-cholesterol (46.35 ± 8.46 mg/dl *vs.* 61.71 ± 15.34 mg/dl) were significantly lower in the taurine-supplemented group than in the unsupplemented group. However, there were no significant differences in the concentration of serum triglyceride and HDL-cholesterol between the rats with and without taurine supplementation.

Table 3. Liver weight of rats fed experimental diets

Variables	Control	TSD	Significance
Liver weight (g)	15.20 ± 0.86 (1)	10.93 ± 0.95	□(2)
Body weight (g)	335.78 ± 12.79	329.56 ± 17.93	NS (3)
Liver wt (g)/bw (g) (4)	0.0047 ± 0.05	0.033 ± 0.01	□

(1) Values are means ± SD. (2) * Significantly different at $p<0.05$. (3) NS Not significantly different at $p<0.05$. (4) Liver weight (g)/body weight (g).

Table 4. Plasma glucose and lipid concentrations of rats fed experimental diets

Variables	Control	TSD	Significance
Glucose (mg/dl)	120.55 ± 14.50 (1)	96.97 ± 8.97	□(2)
Total cholesterol (mg/dl)	107.21 ± 15.76	85.82 ± 5.15	□
Triglyceride (mg/dl)	68.81 ± 12.19	64.47 ± 7.89	NS (3)
HDL-cholesterol (mg/dl)	25.20 ± 10.57	26.99 ± 7.78	NS
LDL-cholesterol (mg/dl)	61.71 ± 15.34	46.35 ± 8.46	□
Atherogenic index	3.47 ± 1.48	2.68 ± 1.53	NS

(1) Values are means ± SD. (2) * Significantly different at $p<0.05$. (3) NS Not significantly different at $p<0.05$.

Nanami *et al*. (1996) reported that supplementation of taurine to the high-cholesterol diet significantly reduced the serum total cholesterol and increased the HDL-cholesterol concentrations in rats. Tsuji *et al*. (1980) also reported that taurine had a hypocholesterolemic effect in experimental hypercholesterolemic animal models. Mizushima *et al*. (1996) reported that oral taurine supplementation attenuated the increase in total cholesterol and LDL-cholesterol in healthy men consuming high-fat and high-cholesterol diets. In agreement with their studies, we also found that taurine supplementation significantly reduced the concentrations of plasma cholesterol and LDL-cholesterol.

Recently, Park and co-workers (1998) observed that rats fed high-cholesterol and taurine-supplemented diet had significantly lower plasma concentrations of total cholesterol (31%) and triglyceride (43%) than those fed high-cholesterol diet without taurine. However, our study is not in agreement with the study conducted by Park and co-workers in the point of triglyceride concentration. The concentration of plasma triglycerides was not significantly affected by taurine supplementation in our study. On the other hand, taurine supplementation has resulted in a decrease in plasma triglyceride levels, but not in cholesterol in diabetic rabbits (Tenner *et al*., 2002). A similar lack of effect of taurine on plasma cholesterol levels has been reported (Petty *et al*., 1990). The reason for this discrepancy is unknown. More detailed research is necessary to explain why the effectiveness of taurine in reducing the plasma triglyceride concentration in high-cholesterol fed rats has been different. Therefore, additional studies are needed to confirm the effect of taurine on the plasma triglyceride concentration in rats fed high-cholesterol diet.

The atherogenic index tended to be lower in the taurine-supplemented group than in the unsupplemented group, but there were no significant difference between these groups.

4.4. Liver Lipid Concentrations

Table 6 summarizes the effects of taurine on liver lipid concentrations. The liver triglyceride concentration was significantly higher in the taurine-supplemented rats than in the unsupplemented rats. The concentrations of total cholesterol and HDL-cholesterol in the liver were significantly lower in the taurine-supplemented rats than in the unsupplemented rats.

Table 6. Liver lipid concentrations of rats fed experimental diets

Variables	Control	TSD	Significance
Total cholesterol (mg/g)	16.51 ± 1.15 (1)	10.27 ± 0.54	□(2)
Triglyceride (mg/g)	25.19 ± 1.90	21.54 ± 2.04	□
HDL-cholesterol (mg/g)	8.01 ± 0.71	11.16 ± 1.56	□

(1) Values are means ± SD. (2) * Significantly different at $p<0.05$.

The plasma cholesterol-lowering effects of taurine appear to have been associated with the increase in fecal bile acid excretion. At least two explanations are possible with respect to the increase in fecal bile acid excretion by taurine. (1) An increase in the conversion of cholesterol to bile acids may occur due to an enhancement of the activity of liver cholesterol 7a-hydroxylase, the rate-limiting enzyme for bile acid synthesis. (2)

Conjugation of bile acids may increase due to an enhancement in taurine concentration in the liver, with subsequent increased bile acid secretion (Sugiyama *et al*., 1989). These results suggest possible roles of taurine as a hypocholesterolemic agent in the rats fed high-cholesterol diet.

4.5. Plasma Free Amino Acid Concentrations

The free amino acid pool represents only a small fraction of the total-body free amino acids. The concentrations in the intracellular space are considerably higher than the plasma concentrations (Grimble *et al*., 1998), and the most part of free amino acids are located in muscle tissue. However, plasma free amino acid concentrations might be of great value in reflecting changes in organ nitrogen handling and altered amino acid metabolism. The effects of taurine on the plasma amino acid concentrations in the rats are given in Tables 7 and 8. The rats fed taurine-supplemented diet exhibited normal levels of the essential amino acids, but the level of histidine was significantly lower than in the rats fed the control diet.

Table 7. Plasma concentrations of free essential amino acids in rats fed experimental diets

Variables	Control	TSD	Significance
Histidine (μmol/l)	58.02 ± 9.55 (1)	44.85 ± 2.67	* (2)
Isoleucine (μmol/l)	57.12 ± 9.34	48.71 ± 3.86	NS (3)
Leucine (μmol/l)	89.61 ± 15.17	76.09 ± 5.44	NS
Lysine (μmol/l)	356.50 ± 71.52	280.40 ± 23.56	NS
Methionine (μmol/l)	25.66 ± 5.17	23.43 ± 3.00	NS
Phenylalanine (μmol/l)	37.42 ± 6.06	31.60 ± 1.06	NS
Valine (μmol/l)	102.84 ± 16.34	86.69 ± 6.17	NS
Arginine (μmol/l)	56.23 ± 39.23	48.77 ± 16.50	NS

(1) Values are means ± SD. (2) * Significantly different at $p<0.05$. (3) NS Not significantly different at $p<0.05$.

Table 8. Plasma concentrations of free nonessential amino acids in rats fed experimental diets

Variables	Control	TSD	Significance
Taurine (μmol/l)	87.26 ± 35.12 (1)	120.27 ± 23.43	NS (2)
Serine (μmol/l)	161.25 ± 31.08	125.52 ± 4.20	NS
Glutamic acid (μmol/l)	30.94 ± 5.97	23.73 ± 1.54	NS
Proline (μmol/l)	86.52 ± 21.13	60.45 ± 5.03	□(3)
Glycine (μmol/l)	209.77 ± 49.86	149.90 ± 10.67	NS
Alanine (μmol/l)	245.37 ± 36.95	208.39 ± 18.53	NS
Citrulline (μmol/l)	43.22 ± 7.95	28.73 ± 2.50	□
α-Aminobutyric acid (μmol/l)	7.23 ± 1.06	6.14 ± 1.27	NS
Tyrosine (μmol/l)	43.21 ± 4.27	38.91 ± 4.80	NS

(1) Values are means ± SD. (2) NS Not significantly different at $p<0.05$. (3) * Significantly different at $p<0.05$.

The plasma taurine concentrations were not significantly different between the two groups. However, the concentration of plasma taurine tended to be higher in the taurine-

supplemented group than in the unsupplemented group. The concentrations of proline and citrulline were significantly lower in the taurine-supplemented group than in the unsupplemented group.

Numerous studies suggested that taurine, 2-aminoethanesulfonic acid, has beneficial effects on cholesterol metabolism by improving the effects that hypercholesterolemia exerts (Yokogoshi *et al*., 1999; Murakami *et al*., 2002) The mechanism of taurine action on cholesterol metabolism is unclear. However, the present study suggests that the hypocholesterolemic effects of taurine are due to the enhancement of cholesterol degradation and excretion of bile acids. In the rats fed a high-cholesterol diet, the cholesterol balance is dependent on the catabolism of cholesterol because cholesterol synthesis is abolished in these rats.

Taurine is used for bile acid conjugation and may facilitate bile acid excretion in feces. Moreover, taurine itself may enhance the biotransformation of cholesterol to bile acids. Increased amounts of bile acids may then enhance the clearance of cholesterol out of the body.

4.6. Correlation of Total Serum Cholesterol and Liver Triglyceride With Plasma Taurine Concentration

The plasma taurine concentrations were significantly correlated with plasma total cholesterol (r =-0.76; P = 0.01) and liver triglyceride (r = -0.80; P = 0.05). The amino acid pattern in the present study is similar to that reported in rats by Boomgaardt *et al*. (1969). Taurine supplementation did not cause any characteristic changes in the plasma aminogram pattern (Fig. 1). Taurine supplementation (1.5%) is thought to be safe in rats.

Table 9. Correlation coefficients of plasma total cholesterol concentration and liver triglyceride with plasma taurine concentration

Variables	Taurine	
	r	Significance
Plasma total cholesterol	-0.76	0.01
Liver triglyceride	-0.80	0.05

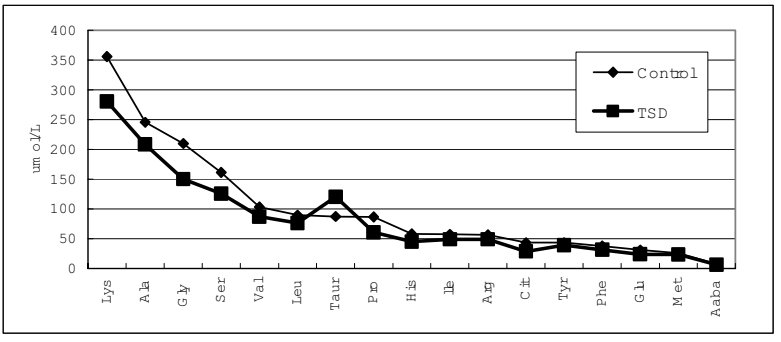

Figure 1. Plasma aminogram of rats fed experimental diets.

5. CONCLUSION

In conclusion, our results show that taurine supplementation decreased the plasma and liver total cholesterol and liver triglyceride levels without modifying the aminogram. These results suggest possible roles of taurine as a hypocholesterolemic agent in rats fed a high-cholesterol diet. Further studies are needed to elucidate the relation of the plasma amino acid concentrations and cholesterol metabolism.

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THE EFFECT OF DIETARY TAURINE SUPPLEMENTATION ON PLASMA AND LIVER LIPID CONCENTRATIONS AND MINERAL METABOLISM IN RATS FED ALCOHOL

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1. ABSTRACT

The purpose of this study was to investigate the effect of dietary taurine supplementation on plasma and liver lipid concentrations and plasma and urine Ca, Mg, Se, Zn concentrations in alcohol-consuming (15% of energy/d) rats. Thirty-two male rats (Sprague-Dawley) were divided into 4 groups and fed experimental diets (non-alchol+control diet, non-alcohol+taurine, alcohol+control diet, alcohol+taurine) for six weeks. The level of taurine supplementation was 1.5 g/100 g diet.

There were no significant differences in the body weight gain and total calorie intake between the control and alcohol groups, but the food efficiency ratio was higher in the control group. The concentrations of plasma total cholesterol, triglyceride, and HDL-cholesterol were higher in the alcohol group, while the levels were apparently reduced in the groups fed taurine. Glutamate-oxaloacetate transaminase (GOT) and glutamate-pyruvate transaminase (GPT) activities were not significantly influenced by alcohol (15% of energy) consumption and dietary taurine supplementation. The plasma concentrations of Ca, Mg, Se, and Zn were not affected by alcohol consumption or dietary taurine supplementation. Only the urinary excretion of Se significantly increased by alcohol consumption, which was decreased by dietary taurine supplementation. These results indicate that taurine exerted some beneficial effects on hypocholesterlemia, hypotriglyceridemia and urinary Se excretion caused by alcohol consumption.

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2. INTRODUCTION

Coronary heart disease is the leading cause of death in the U.S. and most developed countries. The effect of alcohol intake on plasma lipids and coronary heart disease has been the subject of extensive research. Both case-control and cohort studies have described a J or U-shaped association between alcohol intake and coronary heart disease (McElduff and Dobson, 1997) and between alcohol intake and all-cause mortality (Gronbaek *et al*., 1998). Chronic alcohol intake can produce malnutrition as a result of inadequate dietary intake. Energy from alcohol may replace that from food, leading to an overall decline in nutrient intake. Alcohol intake can disturb gastrointestinal functions, leading to reduced or enhanced absorption of vitamins and minerals (Thomson, 1978). It is well known that the excretion of trace minerals is great in persons with chronic alcoholism.

Several studies showed that chronic alcoholism leads to osteopenia and increased incidence of skeletal fractures (Laitinen and Välimäki, 1991; Bikle 1993). Alcohol has been shown to decrease the bone formation rate by decreasing the osteoblast number, osteiod formation, and osteoblast proliferation (Klein *et al.,* 1996).

Magnesium has been suggested to be beneficial in counteracting all phases of the harmful processes that lead to death from ischemic heart disease. Seelig and Heggtveit (1974) found that a low-magnesium diet sometimes contributed to the production of aortic lipidosis in rats fed an atherogenic diet.

Taurine, 2-aminoethanesulfonic acid, is one of the most abundant free amino acids in animal cells and tissues and is thought to have functions in antioxidation, anti-inflammation, osmoregulation, and nerve regulation. Since high serum cholesterol is one of the major risk factors for atherosclerosis and coronary heart diseases, taurine is thought to prevent the development of atherosclerosis.

The effect of alcohol intake on plasma lipids and coronary heart disease has been the subject of extensive research. However, the effect of taurine in animal models of chronic alcoholism has not been studied. The purpose of this study was to investigate the effect of dietary taurine supplementation on the plasma and liver lipid concentrations and the plasma and urine Ca, Mg, Se, and Zn concentrations in alcohol-consuming (15% of energy/d) rats.

3. MATERIALS AND METHODS

Thirty-two male Sprague-Dawley rats (Biogenomics, Seoul) weighing about 170 g were divided into 4 groups of 8 rats in each and fed experimental diets (non-alchol+control diet, non-alcohol+taurine, alochol+control diet, alcohol+taurine) for six weeks. The level of taurine supplementation was 1.5 g/100 g diet. In the alcohol-diet group of rats, the addition of 5% of ethanol in distilled water provided 15% of energy from alcohol in the total energy intake. For the control-diet group, isocaloric sucrose solution was provided instead of 5% ethanol. The compositions of the experimental diets are shown in Table 1. The rats were individually housed in stainless steel cages in a room with controlled temperature (23°C) and humidity (55%) and were given free access to the experimental diets and water. The rats were maintained in a 12-h light (07:00–19:00 h) and dark cycle.

Table 1. Composition of experimental diets (g/100 g diet)

Ingredient	Dietary group	
	Control	Taurine
Casein [(1)]	20	20
Corn starch [(2)]	66	64
Vitamin mixture [(3)]	1	1
Mineral mixture [(4)]	3.5	3.5
A-cellulose [(5)]	5.0	5.0
Choline [(6)]	0.2	0.2
Corn oil [(7)]	5	5
Taurine [(8)]		1.5

(1) Lactic casein, 30 mesh, New Zealand Dairy Board, Willington, NZ. (2) Corn starch, Doosan Co. 234-17 Maam-Ri, Bubal-Eup, Inchon-City, Kyunggi-Do. (3) Vitamin mixture (AIN-76A), supplied by US Corning Laboratory Services Company, Teklad Test Diets, Madison, Wisconsin, Biological Test Material No. 40077. (4) Mineral mixture (AIN-76), supplied by US Corning Laboratory Services Company, Teklad Test Diets, Madison, Wisconsin, Biological Test Material No. 170915. (5) Cellulose, supplied by Sigma Chemical Company, No. C8002. (6) Choline, supplied by Sigma Chemical Company. No. C1629. (7) Corn oil, Shindong-bang oil Co., 4-2 Yang Pyung-Dong, Youngdongpo-Gu Seoul, KSH 2102. (8) Taurine, Dong-A Pharm. Co., Ltd. 434-4 Moknae-dong, Ansan-City, Kyunggi-Do.

On the last day of the experimental period, a blood sample was collected from the abdominal aorta. Plasma was separated from blood by centrifugation (1600×g, 15 min, 4^{o}C) and stored at –70^{o}C until the analysis. After blood collection, the liver was immediately removed, washed with cold saline (9 g NaCl/l), blotted dry on filter paper, weighed, and stored at – 70^{o}C until the analysis.

Plasma lipids (total cholesterol, HDL-cholesterol and triglycerides) were determined by using commercial kits (Wako Pure Chemical, Osaka). The LDL-cholesterol concentrations were estimated with the equation of Friedewald *et al.* (1972). About 2 g of the liver was homogenized and lipids were extracted with chloroform-methanol mixture (2:1. v/v) as described by Folch *et al.* (1957). The concentrations of cholesterol and triglycerides in the liver were enzymatically determined with a commercial kit (Wako Pure Chemical, Osaka).

All laboratory reagents used for the determination of minerals were prepared with distilled, deionized water. Calcium, zinc, magnesium and selenium in plasma and urine were measured by inductively coupled plasma-atomic emission spectrometer (ICP, Jobin-Yvon, Inst. Co., France) after wet digestion of aliquots of freeze-dried material with nitric and perchloric acids.

The statistical significance of differences among the groups was evaluated by two-way ANOVA, using a computer software package (version 8.12; SAS Institute Inc, Cary, NC). The individual comparisons were made by Duncan's multiple range test using ANOVA. Differences were considered to be significant at $p<0.05$. Data are expressed as means ± SD.

4. RESULTS AND DISCUSSION

4.1.Weight Gain and Food Efficiency Ratio

Table 2 shows the weight at beginning, weight at sacrifice, weight gain, food intake, and food efficiency ratio (FER) of rats fed experimental diets. The body weight was not significantly different between the groups at the beginning of the experiment. The body weight gain and food intake of the rats fed alcohol (15% of energy) did not differ from those of the rats fed the control diet. The body weight gain and food intake of the rats fed the experimental diet (taurine 15 g/kg diet) did not differ from those of rats with or without alcohol. The FER was significantly lower in the rats fed alcohol diet than in the rats fed control diet.

Table 2. Body weight, FER, and total energy intake of fed the experimental diets

Variables	Non-alcohol		Alcohol	
	Control	Taurine	Control	Taurine
Initial body weight (g)	173.1 ± 11.81$^{(a1)}$	167.7 ± 7.74$^{(a)}$	176.3 ± 14.18$^{(a)}$	68.3 ± 6.77$^{(a)}$
Final body weight (g)	415.43 ± 39.72$^{(a)}$	429.50 ± 56.28$^{(a)}$	436.18 ± 32.77$^{(a)}$	442.06 ± 33.31$^{(a)}$
FER $^{(2)}$	0.24 ± 0.01$^{(a)}$	0.25 ± 0.05$^{(a)}$	0.21 ± 0.01$^{(b)}$	0.20 ± 0.02$^{(b)}$
Total energy intake (kCal/day)	113.90 ± 8.81$^{(a)}$	116.13 ± 9.12$^{(a)}$	113.28 ± 5.36$^{(a)}$	119.37 ± 9.86$^{(a)}$

(1) Values are means ± SD. (2) FER (food efficiency ratio) = weight gain (g)/food intake (g). (3) Values within row with different superscripts are different at $p<0.05$ by Duncan's multiple test.

4.2. Blood and Hepatic Lipids and GOT and GPT

The effects of alcohol and taurine supplementation on blood lipids are presented in Table 3. The alcohol consumption significantly increased the plasma total cholesterol concentration. Taurine significantly lowered the plasma total cholesterol concentration in the rats fed alcohol or without alcohol. The alcohol consumption significantly increased the plasma triglyceride concentration.

Table 3. Plasma lipid concentrations of rats fed the experimental diets

Variables	Non-alcohol		Alcohol	
	Control	Taurine	Control	Taurine
Total cholesterol (mg/dl)	70.68 ± 10.18$^{(a1)}$	53.34 ± 11.45$^{(b)}$	103.14 ± 12.42$^{(c)}$	87.13 ± 8.23$^{(d)}$
Triglyceride (mg/dl)	49.71 ± 13.67$^{(a)}$	30.36 ± 3.99$^{(a)}$	103.65 ± 43.36$^{(b)}$	51.30 ± 12.47$^{(a)}$
HDL-cholesterol (mg/dl)	45.45 ± 4.28$^{(a)}$	34.05 ± 5.36$^{(b)}$	37.29 ± 4.74$^{(b)}$	33.27 ± 5.88$^{(b)}$
LDL-cholesterol (mg/dl)	17.62 ± 7.68$^{(a)}$	15.34 ± 14.94$^{(a)}$	48.46 ± 14.29$^{(b)}$	44.98 ± 15.33$^{(b)}$
Atherogenic index	0.58 ± 0.13$^{(a)}$	0.68 ± 0.50$^{(a)}$	1.80 ± 0.42$^{(b)}$	1.73 ± 0.66$^{(b)}$

(1) Values are means ± SD. (2) Values within row with different superscripts are different at $p<0.05$ by Duncan's multiple test.

Taurine significantly lowered the plasma triglyceride concentration in the rats fed alcohol. It also tended to decrease that in the rats fed alcohol-free diet. The alcohol consumption significantly decreased the plasma HDL-cholesterol concentration in the rats fed alcohol. The alcohol consumption significantly increased the plasma LDL-cholesterol concentration in the rats fed alcohol. However, taurine supplementation did not affect to the plasma LDL-cholesterol concentration in the rats fed alcohol. The rats fed alcohol showed a significantly increased atherogenic index (Table 3).

The concentration of liver triglycerides in the rats fed alcohol was significantly higher than that in those fed the alcohol-free diet. Park *et al.* (1998) have shown that taurine reduced the hepatic triglyceride concentration in rats and Yan *et al.* (1993) that taurine lowered the liver concentration of triglycerides and total cholesterol in rats. In the present study, taurine significantly decreased the concentrations of liver triglycerides. However, the liver total cholesterol concentrations did not differ between the rats fed with or without taurine. The concentration of liver triglycerides in rats fed without or with alcohol was significantly lower than that in those fed the control diet without taurine (Table 4).

There were no significant differences in GOT and GPT between the rats with and without alcohol consumption and taurine supplementation (Table 5).

Table 4. Hepatic lipid concentrations of rats fed the experimental diets

Variables	Non-alcohol		Alcohol	
	Control	Taurine	Control	Taurine
Total cholesterol (mg/dl)	$2.40 \pm 0.51^{(a1)}$	$3.42 \pm 0.72^{(a)}$	$3.82 \pm 0.96^{(a)}$	$4.81 \pm 1.44^{(a)}$
Triglyceride (mg/dl)	$6.80 \pm 2.00^{(a)}$	$4.61 \pm 0.84^{(b)}$	$8.87 \pm 1.87^{(c)}$	$5.88 \pm 1.36^{(a)}$

(1) Values are means ± SD. (2) Values within row with different superscripts are different at $p<0.05$ by Duncan's multiple test

Table 5. Activity of glutamate-oxaloacetate transaminase (GOT) and glutamate-pyruvate transaminase (GPT) in plasma

Variables	Non-alcohol		Alcohol	
	Control	Taurine	Control	Taurine
GOT (U/l)	$74.95 \pm 16.78^{(a1)}$	$68.82 \pm 19.93^{(a)}$	$76.65 \pm 20.31^{(a)}$	$66.36 \pm 12.72^{(a)}$
GPT (U/l)	$26.41 \pm 7.86^{(a)}$	$24.97 \pm 4.21^{(a)}$	$27.85 \pm 6.24^{(a)}$	$27.89 \pm 2.83^{(a)}$

(1) Values are means ± SD. (2) Values within row with different superscripts are different at $p<0.05$ by Duncan's multiple test.

4.3.Plasma Ca, Zn, Mg, and Se

The effects of alcohol and taurine on plasma trace minerals are presented in Table 6. There were no significant differences in the plasma Mg, Zn, Se, and Ca concentrations among the groups. The plasma electrolytes were not affected by alcohol consumption or taurine supplementation. In a human study, the mean plasma selenium level and mean

Table 6. Plasma levels of Ca, Mg, Se and Zn in rats

Variables	Non-alcohol		Alcohol	
	Control	Taurine	Control	Taurine
Ca (mg/dl)	5.41 ± 2.07$^{(a1)}$	4.26 ± 1.86$^{(a)}$	4.94 ± 2.33$^{(a)}$	4.32 ± 1.76$^{(a)}$
Mg (mg/dl)	7.62 ± 1.90$^{(a)}$	5.70 ± 3.23$^{(a)}$	7.81 ± 3.76$^{(a)}$	6.09 ± 3.81$^{(a)}$
Se (μg/l)	1.08 ± 0.07$^{(a)}$	1.07 ± 0.10$^{(a)}$	2.67 ± 3.82$^{(a)}$	0.96 ± 0.09$^{(a)}$
Zn (μg/l)	5.13 ± 1.96$^{(a)}$	5.58 ± 2.85$^{(a)}$	5.43 ± 2.13$^{(a)}$	5.12 ± 2.01$^{(a)}$

(1) Values are means ± SD. (2) Values within row with different superscripts are different at $p<0.05$ by Duncan's multiple test.

urinary excretion of selenium were both significantly lower in alcoholic subjects when compared to the controls (Dutta *et al*., 1983). Furthermore, the daily dietary intake of selenium was estimated to be below the recommended safe and adequate range in the majority of the alcoholic subjects.

The present research suggests that selenium depletion does occur in alcoholic subjects most likely due to poor dietary intake (Dutta *et al*., 1983). Selenium, an essential trace nutrient, has been reported to improve immune functions and ameliorate specific disease conditions in humans and animals (Levander, 1987). More recently, convincing evidence has been presented that consumption of Se in amounts up of 3–5 times the recommended dietary allowance may prevent certain cancers, including colon cancer (Clark *et al*., 1996). Total cancer incidence and mortality were significantly reduced by Se supplementation, with specific reductions of the relative risk for lung, prostate, and colorectal cancer.

4.4. Urinary Ca, Mg, Se, and Zn Excretion

The effects of alcohol consumption and taurine supplementation on urinary loss of calcium, magnesium, zinc and selenium are shown in Table 7. Alcohol consumption had no significant effect on urinary calcium, magnesium and zinc excretion. However, there was an apparently greater, although not statistically significant, difference between the alcohol and non-alcohol groups in case of calcium, when compared with the differences in the other minerals.

This may be explained by one individual animal in the chronic exposure of alcohol, which had a low amount of bone mineral content. The long-term consequence of a small change in the calcium balance is substantial. A small increase in urinary calcium loss per day will result in a moderate amount of loss for a long period.

Table 7. Urinary excretion of Ca, Mg, Se and Zn in rats

Variables	Non-alcohol		Alcohol	
	Control	Taurine	Control	Taurine
Ca (mg/day)	0.44 ± 0.26$^{(a1)}$	0.25 ± 0.13$^{(a)}$	0.50 ± 0.16$^{(a)}$	0.35 ± 0.11$^{(a)}$
Mg (mg/day)	0.29 ± 0.23$^{(a)}$	0.11 ± 0.74$^{(a)}$	0.31 ± 0.45$^{(a)}$	0.23 ± 0.16$^{(a)}$
Se (μg/day)	0.95 ± 0.08$^{(a)}$	0.82 ± 0.07$^{(b)}$	1.11 ± 0.28$^{(c)}$	0.93 ± 0.05$^{(a)}$
Zn (μg/day)	5.50 ± 5.48$^{(a)}$	3.33 ± 2.74$^{(a)}$	5.57 ± 2.13$^{(a)}$	2.90 ± 2.01$^{(a)}$

(1) Values are means ± SD. (2) Values within row with different superscripts are different at $p<0.05$ by Duncan's multiple test.

Selenium is a constituent of glutathione peroxidase, an enzyme that inactivates the toxic hydrogen peroxide and hydroperoxides. Selenium is identified as an essential antioxidant nutrient. The direct effects of alcohol on bone and mineral metabolism have been described in both rats and men. Studies on chronic alcohol consumption in growing male and female rats have indicated that bone growth is suppressed, leading to a failure to acquire a normal bone mass (Turner *et al.,* 1987).

The bone loss in adult rats fed *ad libitum* a liquid diet containing increasing concentrations of ethanol until receiving the appropriate percentage of total caloric intake, resulted in a dose-dependent decrease in the trabecular thickness, bone turnover and bone formation rate (Turner *et al*., 2001). Alcohol may also have deleterious effects on the bone homeostasis through increased excretion of calcium and magnesium (Kalbfleisch *et al*., 1963). However, a moderate intake of alcohol did not affect bone density (Felson *et al*., 1995).

The urinary excretion of selenium was significantly higher in the alcohol group of rats fed the control diet than in the non-alcohol group of rats fed the control diet. The urinary selenium excretion was 0.95 ± 0.08 and 1.11 ± 0.28 μg/d in the non-alcohol group of rats fed the control diet and the alcohol group of rats fed the control diet, respectively. The urinary excretion of selenium was significantly lower in the taurine-supplemented rats than in the non-supplemented rats. Selenium as an essential component of selenocysteine-containing protein is involved in most aspects of cell biochemistry and function. As such, there is much potential for selenium to influence the immune system. A correlation between the lack of Se in food and different diseases such as cardiovascular disease, cancer, rheumatoid arthritis, and cataract has been proposed (Salonen *et al.,* 1984).

5. CONCLUSION

The animals fed alcohol showed significantly higher serum total cholesterol and triglyceride concentrations than those fed the control diet, but the supplementation of 15 g taurine/kg diet significantly reduced the concentration of serum total cholesterol. Taurine significantly lowered the urinary Se excretion in the rats fed alcohol. Our rat model confirmed that consumption of alcohol increases the plasma total cholesterol and triglyceride concentrations. We also confirmed that dietary supplementation of taurine decreased alcohol-induced hypercholesterolemia and hypertriglycedemia in rats. These results indicate that taurine exerts some beneficial effects on hypocholesterlemia, hypotriglyceridemia, and urinary Se excretion caused by alcohol consumption.

6. ACKNOWLEDGMENTS

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EFFECTS OF TAURINE SUPPLEMENTATION ON CHOLESTEROL LEVELS WITH POTENTIAL RAMIFICATION IN ATHEROSCLEROSIS

John B. Lombardini and Julius D. Militante[1]

1. INTRODUCTION

In 2004 the present authors wrote a review on the effects of dietary taurine supplementation and its hypolipidemic and antiatherogenic effects (Militante and Lombardini, 2004). At this time there was considerable information that taurine supplementation could lower cholesterol levels in animal models but there were no follow-up human studies to determine if the results would be beneficial for man. Data were reviewed that demonstrated that the hypercholesterolemic effects of high-fat diets could be reduced by taurine supplementation and that taurine improved cholesterol metabolism and prevented gallstone formation and atherogenesis in mice fed a high-fat diet. Furthermore, it was demonstrated that the rat high-fat diet model provided the most extensive proof of the ameliorating effects of taurine. Taurine supplementation was also shown to be beneficial in the hamster model of hypercholesterolemia by decreasing acyl CoA:cholesterol acyltransferase activity and increasing LDL receptors. However, when the animal model shifted to the rabbit the hypolipidemic effect of taurine was inconsistent, that is, taurine reduced cholesterol levels in some studies while had no effects in other studies.

In this previously published review (Militante and Lombardini, 2004), the data presented in the literature from animals models (mice, rats, rabbits) suggested that there is a genetic predisposition for hyperlipidemia and that taurine is beneficial in these models in reducing either the high cholesterol levels and/or reducing the atherosclerotic lesions that were observed.

Furthermore, in studies on diabetic hyperlipidemia in rats it was demonstrated that of both serum and liver lipid levels that were observed in this model for diabetes (reviewed in Militante and Lombardini, 2004). In these studies, the apparent mechanism

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of action of taurine is to increase bile excretion and thus it was suggested that taurine may be a potential hypolipidemic agent for diabetics. Thus, the authors (Militante and Lombardini, 2004) of the review concluded that there is considerable literature that taurine has specific cardiovascular benefits, especially in pathologic conditions of increased lipid levels that induce atherosclerosis. Obviously, studies on taurine supplementation in humans with elevated cholesterol and potential cardiac pathology are warranted to determine the usefulness of taurine as an hypolipidemic agent as it is known to be a safe when added to the diet and certainly as a dietary supplement would be quite convenient and inexpensive.

2. ADDITIONAL LITERATURE REPORTS ON THE EFFECT OF TAURINE SUPPLEMENTATION IN RAT/MOUSE MODELS WITH HIGH CHOLESTEROL

When taurine (3%) was added to the diets of rats fed diets supplemented with (a) cholesterol, (b) cholestyramine, or (c) sodium cholate, serum cholesterol was significantly reduced in the rats fed diets containing additional cholesterol and sodium cholate but not in the diet supplemented with cholestyramine (Nishimura *et al.*, 2003). In the two diets in which taurine had an effect, hepatic cholesterol 7 alpha hydroxylase activity was increased while this enzyme did not increase in the diet supplemented with cholestyramine. It was also noted in these experiments that fecal bile acid excretion was increased in only rats fed the cholesterol-supplemented diet. These authors suggested that taurine increases hepatic cholesterol 7 alpha hydroxylase activity and consequently reduces the levels of serum cholesterol.

Chen *et al.* (2003) performed the following experiments with mice. Three different diets were tested: (a) control, (b) taurine supplemented (1% added to the food), and (c) taurine deficient (0.5% guanidinoethyl sulfonate, a taurine transport inhibitor, added to the drinking water). In the taurine supplemented group, serum and liver cholesterol and fecal bile acid excretion increased. No differences in the LDL receptor protein levels were observed in either the taurine supplemented group or the taurine deficient group and thus it was suggested that the decrease in the total cholesterol was due to the observed decreases in VLDL + LDL (42% reduction). Triglyceride levels were also decreased in the animals fed the taurine supplemented diet (15% reduction). Gallstone formation was prevented in the taurine supplemented group by promoting both the conversion of cholesterol to bile acid and increasing the fecal excretion of bile acids. On the contrary gallstone formation increased in the taurine deficient animals again supporting the evidence that taurine is necessary for cholesterol catabolism.

Taurine supplementation to stroke-prone spontaneously hypertensive rats fed a high cholesterol diet also resulted in a lowering of both serum cholesterol levels and arterial fat deposition (Yamori *et al.*, 2004). It was suggested that the serum cholesterol levels were regulated in the taurine supplemented animals due to a taurine affect on the gene expression of 7 alpha-hydroxylase, the rate-limiting step of bile acid formation. The antioxidative effects of taurine were thought to be antiatherogenic in this animal model by removing hypochlorous acid and inhibiting the production of oxidized LDL. Overall in the taurine supplemented group of rats the development of hypertension and stroke was decreased.

3. EFFECTS OF TAURINE SUPPLEMENTATION IN RABBIT MODELS WITH HIGH CHOLESTEROL

In searching the literature and as noted in the review that was written in 2004 (Militante and Lombardini, 2004), it is quite clear that the effects of taurine in lowering cholesterol in rabbit models are inconsistent. However, the experiments by Balkan *et al.* (2002) show that taurine (2.5%) added to the diets of rabbits fed a high cholesterol diet for 2 months reduced the plasma and the erythrocyte cholesterol levels by 22% and 19%, respectively. The effect of supplemental dietary taurine was also tested for its protective effects on erythrocyte hemolysis in the high cholesterol diet. Taurine has no effect on hemolysis, remaining the same in both situations: high cholesterol diet = 27.5% versus high cholesterol diet supplemented with taurine = 25.2%. Hydrogen peroxide-induced lipid peroxidation did not change in the animals fed a high cholesterol diet compared to a control normal diet and taurine supplementation did not affect the erythrocyte lipid peroxidation.

In a subsequent series of experiments Balkan and colleagues (2004) tested whether taurine (1% in drinking water) had any ameliorating effects on atherosclerotic lesions and lipid peroxidation in rabbits fed a high cholesterol diet for 8 months [0.5% cholesterol for 3 months, 0.25% cholesterol for 5 months, 4 months (regression period) on a normal diet +/- taurine supplementation]. In the aorta, the atherosclerotic lesions were decreased in the taurine-treated animals fed a high cholesterol diet compared to the animals fed high cholesterol diets without taurine. Plasma and aorta malondialdehyde (MDA) and diene conjugate (DC) formation did not change in the regression period with or without taurine supplementation. However, MDA and DC formation were attenuated in the liver by taurine treatment: MDA decreased 17% with taurine; DC formation decreased 17% with taurine.

4. HUMAN STUDIES ON THE EFFECTS OF TAURINE SUPPLEMENTATION ON ELEVATED LIPID LEVELS

While there is ever increasing documentation that taurine has a cholesterol-lowering effect and an antiatherogenic effect in various animal models which are made hypercholesteremic due to a high fat intake, there is a paucity of information as to the effect of taurine in human subjects.

Studies by Zang *et al.* (2004) attempted to answer the clinical question as to whether taurine could benefit individuals with high cholesterol levels. In these studies, 30 young college students who were overweight or obese were divided into 2 equal groups designated as the placebo group or the taurine group. Taurine (3 g/day) or placebo was administered orally for 7 weeks. The following clinical parameters were measured: triacylglycerol (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and plasma glucose (G). The atherogenic index (AI) was also calculated. Both TG and the AI were both decreased after 7 weeks taurine supplementation. TG was decreased by 16% and the AI was decreased by 16%. Interestingly, the body weight of those individuals supplemented with taurine decreased by 2%. The authors concluded from their data that supplemental dietary taurine may be useful in improving lipid metabolism in overweight individuals and prevent cardiovascular disease.

5. CONCLUSIONS

It is quite convincing from the literature that taurine supplemented in the diet and administered to various animal models (rat, mouse, and rabbit) has a significant effect in reducing both total cholesterol and the induced atherogenic lesions. Unfortunately, there are very few studies in which taurine has been administered to humans. Because of the present prevalence of high fat diets in our society and the serious sequellae associated with high serum cholesterol levels, it is considered after reviewing the data in the literature that more studies are needed to access the value of supplemental dietary taurine in the human model. If truly beneficial in lowering human hypercholesterol levels, taurine could be an inexpensive, nontoxic dietary supplement with significant cardiovascular properties.

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Part 4.
Taurine in Heart and Muscles

MOLECULAR MECHANISMS OF CARDIOPROTECTION BY TAURINE ON ISCHEMIA-INDUCED APOPTOSIS IN CULTURED CARDIOMYOCYTES

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1. INTRODUCTION

An integral part of the pathogenesis of heart failure is myocyte loss. The traditional explanation for myocyte loss was cell necrosis but there has been a surge of evidence affirming the role of apoptosis in the genesis of heart failure (Garg *et al.,* 2005). Evidence for apoptotic cell death was shown in clinical cases of myocardial infarction, as well as in rabbit, rat, and mouse models of continuous ischemia or ischemia/reperfusion (Garg *et al.,* 2005). It has been shown that the mitochondrial pathways participate in apoptosis induced by ischemia (Garg *et al.,* 2005). Taurine (2-aminoethanesulfonic acid), the β-amino acid, is one of the factors that regulates the degree of apoptosis during ischemia (Roysommuti *et al.,* 2003; Schaffer *et al.,* 2003). However, little is known about the cytoprotective signalling pathways mediate this response. We have previously reported that isolated neonatal cardiomyocytes become resistant to ischemia-induced apoptosis when exposed to medium containing 20 mM taurine (Takahashi *et al.,* 2003). In this study, the interaction between taurine and mitochondria-mediated apoptosis is investigated in a newly developed simulated ischemia model utilizing isolated cardiomyocytes that are incubated with medium containing and lacking taurine and then sealed within cultured flasks (Takahashi *et al.,* 2003, Takatani *et al.,* 2004a,b).

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2. MATERIALS AND METHODS

2.1 Cell Cultures and the Newly Simulated Ischemia Model

Primary cardiomyocyte cultures from 1-day–old Wistar rats were prepared according to the procedure described previously (Takahashi *et al.,* 2001). All experimental procedures were approved by the Animal Care Committee of Osaka University and conformed to international guidelines. The simulated ischemia model mimics the clinical stresses of ischemia, including the stresses of hypoxia, acidosis, and stagnant incubation medium (Takahashi *et al.,* 2001).

2.2. Detection of Mitochondrial Dysfunction and Ischemia-Induced Apoptosis of Cardiomyocytes

Evaluation of apoptosis was performed with a fluorescent dye, Hoechst 33258, and a commercially available cell death detection kit to find DNA strand breaks using the terminal deoxynucleotidyl transferase-mediated dUDP nick-end labelling (TUNEL) reagent. Loss of mitochondrial membrane potential ($\Delta\psi$) was assessed using a fluorescent dye, the lipophilic cationic probe JC-1(5,5',6,6'-tetrachloro-1,1',3,3'- tetraethylbenzimid-azolylcarbonyanine). Cells were incubated with JC-1 and examined with an Olympus fluorescence microscope.

2.3. Western Blot Analysis

Proteins from the mitochondrial fraction, cytosolic fraction and the total cell lysates were analyzed by SDS-PAGE (12.5% or 14% gel). After blotting, the Immobilon-P membrane (Millipore) was blocked with 5% bovine serum albumin (BSA) in Tween 20-containing phosphate-buffered saline (PBS) at room temperature for 1 hour. Immunoblots were incubated at room temperature for 60 minutes with the specific primary antibody to anti-cytochrome c antibody, anti-Bax, anti-Bcl-2, anti-p53, Apaf-1, caspase-3, -9, Akt, phosphorylated Akt or hemagglutinin (HA). After further washing, the membrane was incubated for 60 minutes with the secondary antibody (horseradish peroxidase-conjugated). The ECL reaction was used for detection. Blots were reprobed with anti-actin antibody as a loading control. Quantitative analysis of immunoblotted bands was performed by computer program (NIH Image, Version 1.61).

2.4 Adenovirus

The recombinant replication-defective adenovirus expressing a dominant-negative form of Akt (dnAkt) was prepared as described Fujio et al. (2000).

2.5. Statistical Analysis

Statistical evaluation of data was performed either with Student's t test, χ^2-test or analysis of variance with the Bonferroni method used to compare individual data points for a significant F value. Each value was expressed as mean ± SEM. Differences were considered significant when the calculated p value was <0.05.

3. RESULTS AND DISCUSSION

3.1. The Mitochondrial Apoptotic Pathway in Simulated Ischemia Model

A cellular and biochemical approach was used to elucidate the mechanism underlying cellular apoptosis in an in-vitro cell culture model of sealing-induced cardiac ischemia, which mimics distinct features of ischemic injury (Fig. 1). Ischemia was induced by the sealing procedure for 24-72 h and resulted in cardiac apoptosis, as evidenced by an increase in DNA fragmentation (nucleic staining by Hoechst 33258 and TUNEL) and internucleosomal cleavage of DNA (Takahashi *et al.,* 2001, 2003; Takatani *et al.,* 2004b). Simulated ischemia also caused mitochondrial dysfunction resulting in both cytochrome c release from the mitochondria and mitochondrial depolarization. In support of the notion that cytochrome c release can be mediated by pro-apoptotic members of the Bcl-2 family, it was found that simulated ischemia-induced apoptosis was accompanied by alterations in the Bax/Bcl-2 protein ratio and the expression of p53 within 24 h of ischemia (Fig. 2B,C). Based on the appearance of active forms after 30 h of ischemia, the apoptotic cascade appeared to be initiated by the activation of caspase-9/-3 (Fig. 3). This evidence demonstrated a mechanistic link between mitochondria and apoptosis in an in-vitro cell culture model of sealing-induced cardiac ischemia (Takahashi *et al.,* 2000, 2001, 2003; Takatani *et al.,* 2004b).

3.2. The Anti-Apoptotic Effect of Taurine Through the Mitochondrial Pathway of Apoptosis

One of the factors that regulate the degree of apoptosis during ischemia is the amino acid taurine. To study the mechanism underlying the beneficial effect of taurine, the interaction between taurine and mitochondria-mediated apoptosis was examined using a simulated ischemia model with cultured rat neonatal cardiomyocytes sealed in closed flasks. Exposing the cells to medium containing 20 mM taurine reduced the degree of apoptosis following periods of ischemia varying from 24-72 h (Takahashi *et al.,* 2003; Takatani *et al.,* 2004b). As shown in Fig. 2A, taurine significantly decreased apoptosis from 42% to 15 % after a 72-h ischemic insult. Although taurine treatment was cardioprotective, it had no effect on mitochondrial membrane potential and cytochrome c release (Takatani *et al.,* 2004b). Moreover, cells placed in medium containing 20 mM taurine showed no change in the levels of the apoptosis-related proteins, such as Bax Bcl-2 and p53, during simulated ischemia (Fig. 2B,C). However, we demonstrated that taurine prevented the ischemia-induced apoptosis in cardiomyocytes, accompanied by the inactivation of caspase-9 and -3 (Takatani *et al.,* 2004a,b) (Fig. 3A).

In untreated cells, simulated ischemia facilitated the formation of the oligometric Apaf-1/caspase-9 apoptosome, as evidenced by the 60% increase in the amount of caspase-9 associated with Apaf-1 during ischemia (Takatani *et al.,* 2004b). Taurine loading also suppressed the formation of the Apaf-1/caspase-9 apoptosome and the interaction of caspase-9 with Apaf-1 (Fig. 3A) (Takatani *et al.,* 2004a). These findings demonstrate that taurine effectively prevents myocardial ischemia-induced apoptosis by inhibiting the assembly of the Apaf-1/caspase-9 apoptosome.

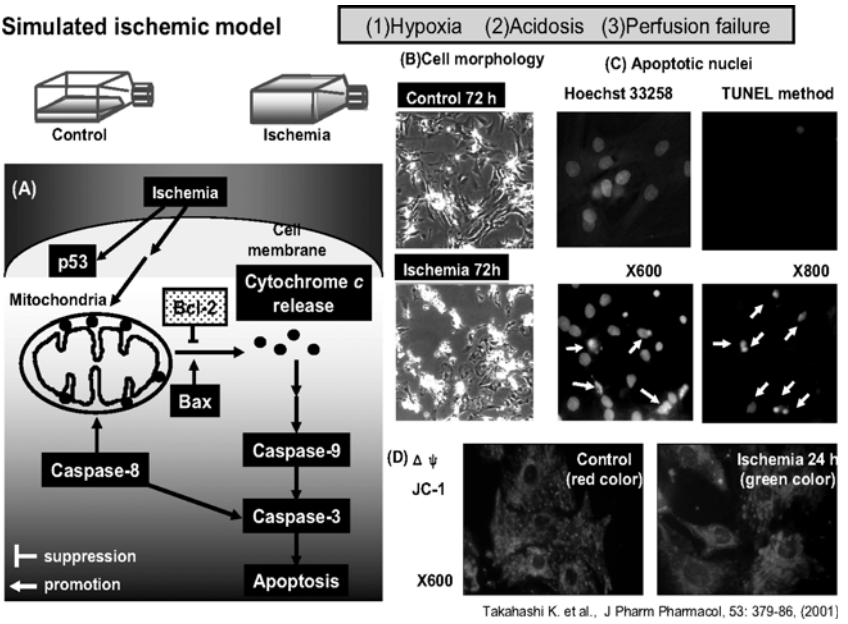

Figure 1. Characteristics of simulated ischemia model. (A) The mitochondrial apoptotic pathway in simulated ischemic model; representative microphotographs of (B) cell morphology, (C) apoptotic nuclei by Hoechst 33258 or TUNEL staining, and (D) mitochondrial membrane potential (Δψ) determined using the potential-sensitive fluorescent probe JC-1.

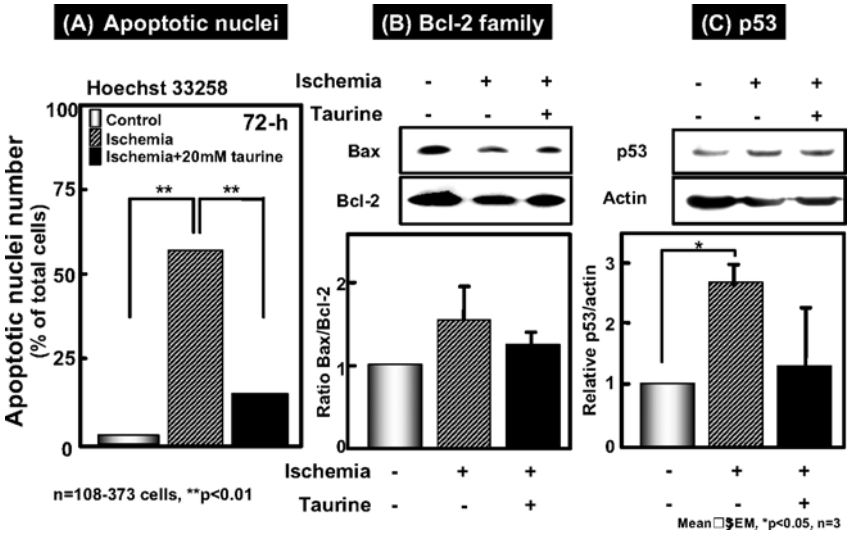

Figure 2. The anti-apoptotic effect of taurine and the expression of the apoptotic-related proteins by Western blot analysis in cardiomyocytes exposed to simulated ischemia. (A) Percentage of cells undergoing apoptosis as measured by Hoehst 33258 stain. (B) Quantative analysis of Bcl-2 and Bax expression in mitochondria exposed to ischemia for 24 h. (C) Quantitative Western blot analysis of p53 expression in cardiomyocytes exposed to simulated ischemia for 3 h. The blots were re-probed with anti-actin as a loading control. *p<0.05 *vs.* control group.

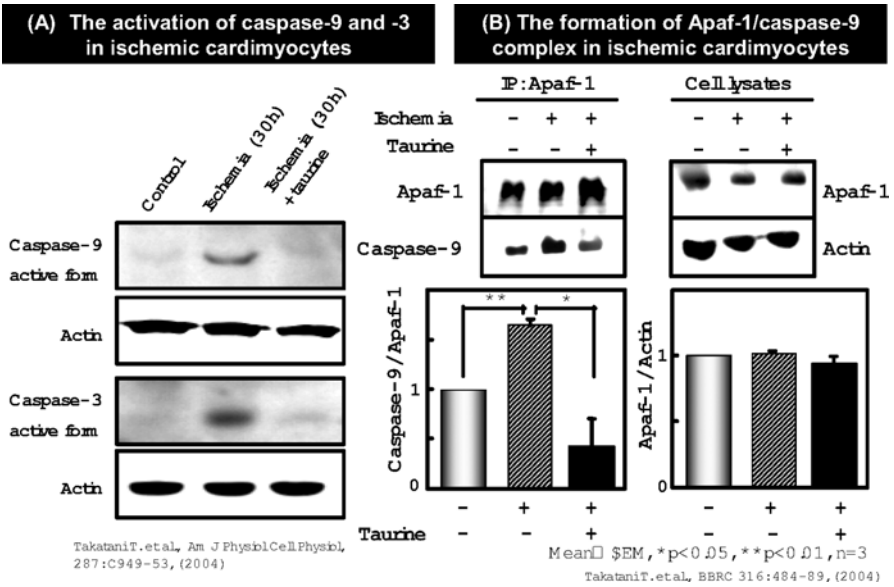

Figure 3. Effects of taurine on the activation of caspase-9 and –3 (A) and the formation of Apaf-1/caspase-9 complex (B) in ischemic cardiomyocytes. (A) Active subunits p35 and p17 of caspase-9 and caspase-3, respectively, were assessed by immunoblot analysis using antibody against either caspase-9 (top) or caspase-3 (bottom). (B) An equal volume of cell lysate was immunoprecipitated (IP) with antibody against Apaf-1, and the precipitate, as well as the cell lysate, was analyzed by immunoblot with antibody against Apaf-1 or caspase-9.

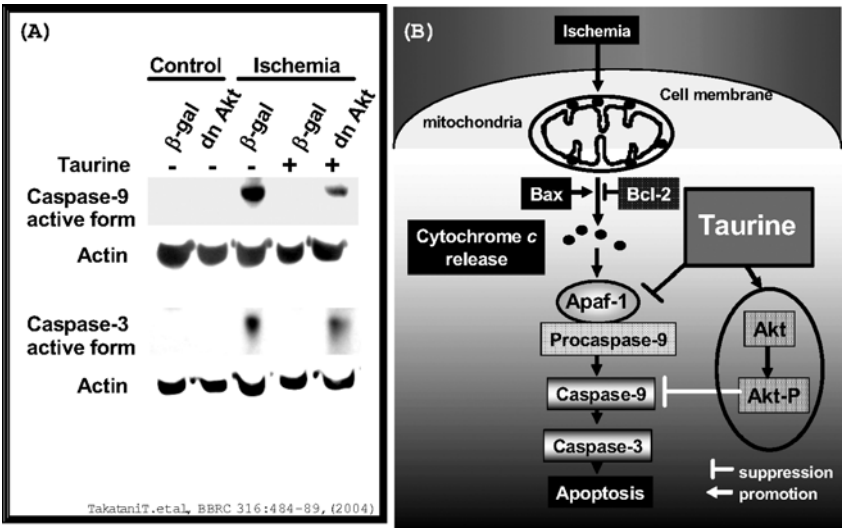

Figure 4. (A) Akt-linked protective effect of taurine in simulated ischemia-induced apoptosis. Cardiomyocytes were infected overnight with adenovirus vectors expressing β-gal, or dn Akt at an MOI of 25. (B) Regulation of ischema-induced apoptosis by taurine in cardiomyocytes.

3.3. Akt-Linked Protective Effect of Taurine Against Simulated Ischemia-Induced Apoptosis

Akt has been demonstrated to inhibit apoptosis in cardiomyocytes. In this study, to investigate whether taurine-mediated cytoprotection correlates with the Akt pathway, isolated cardiomyocytes were subjected to a newly developed simulated ischemia model. Taurine (20 mM) treatment attenuated simulated ischemia-induced decline in the activity of Akt (Takatani *et al.,* 2004a). Activated Akt kinase has been proposed to play a central role in suppressing apoptosis by modulating the activities of Bcl-2 family proteins, and/or caspase-9. Interestingly, although taurine treatment had no effect on the expression of Bcl-2 in mitochondria and the level of cytosolic cytochrome c, it inhibited ischemia-induced cleavage of caspases-9 and -3. Taurine has been shown to regulate myocardial calcium homeostasis through either direct or indirect modulation of several key calcium transporters (Roysommuti *et al.,* 2003). Since calcium and oxidative stress regulate the activation of Akt, there is reason to suspect that taurine might benefit the cardiomyocyte through the Akt-linked pathway.

To further determine whether Akt is essential for taurine-mediated cytoprotection, adenovirus vectors expressing dnAkt were transfected into cardiac myocytes. Transfection with adeno-ß gal, the control vector, showed no detectable effect on taurine-induced decrease in apoptotic cells during ischemia, however transfection with dnAkt eliminated the effect of taurine. Furthermore, as shown in Fig. 4A, taurine prevented the ischemia-induced increase in caspase-9 and caspase-3 activities, which was also abrogated by the transfection of dnAkt. Namely, adenoviral transfection with dominant negative Akt attenuated taurine-mediated anti-apoptotic activity, blocking the taurine-mediated suppression of caspase-9 and 3 activation (Takatani *et al.,* 2004a).

4. CONCLUSION

We have identified a mechanistic link between taurine-mediated cytoprotection and the Akt/caspase-9 pathway (Figure 4B). First, taurine significantly attenuates ischemia-induced apoptosis, an effect associated with Akt activation. Second, taurine does not inhibit cytochrome c release from the mitochondria but prevents caspase-9/3 activation. Third, taurine treatment inhibits Apaf-1/caspase-9 apoptosome formation under ischemic conditions. However, adenovirus transfer of the dominant negative form of Akt abrogates both taurine-cytoprotection and suppression of caspase-9 and -3 activation. These findings provide the first evidence that taurine prevents ischemia-induced apoptosis in cardiomyocytes by inhibiting Apaf-1/caspase-9 apoptosome formation and promoting Akt-mediated caspase-9 inactivation.

5. ACKNOWLEDGMENTS

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MYOGENIC INDUCTION OF TAURINE TRANSPORTER PREVENTS DEXAMETHASONE-INDUCED MUSCLE ATROPHY

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1. INTRODUCTION

Taurine is present in high concentrations in excitable tissues, i.e. heart and skeletal muscle, and has many physiological actions such as membrane stabilization, osmoregulation and antioxidation (Kendler, 1989; Schaffer *et al*., 2000; Fang *et al*., 2002). In skeletal muscles, taurine plays a role in excitation-contraction coupling, possibly through the modulation of ion movements and calcium homeostasis (Huxtable, 1992; De Luca *et al*., 1996). Furthermore, numerous evidences show that taurine protects skeletal muscles against damage. For example, it has been reported that taurine treatment suppressed muscle cramp and fatigue by exercise (Yatabe *et al*., 2003) and ischaemia–reperfusion injury (Oz *et al*., 1999; McLaughlin *et al*., 2000; Guo *et al*., 2002).

While plasma taurine level ranges about 20-100 μM, the intracellular level in skeletal muscle is about 60 mM, which is significantly higher than in non-excitable tissues (Huxtable, 1992). This gradient and high concentration of taurine is possibly maintained by the uptake from plasma, mainly via taurine transporter (TauT). Indeed, taurine content in skeletal muscle was markedly decreased by guanidinethanesulfonate (GES), the inhibitor of taurine uptake, and by disruption of TauT in mice (Warskulat *et al*., 2004). Interestingly, knockout of *TauT* gene in mice results in the decrease of total exercise capacity and the impairment of skeletal muscle (Warskulat *et al*., 2004), suggesting that TauT is necessary for development and/or function of mature skeletal muscle. However, the regulatory mechanism for TauT expression and its responsibility for sustaining high amount of taurine in skeletal muscle have not been clarified.

To reveal the regulatory properties in skeletal muscle, we investigated the regulation of TauT expression during myogenic differentiation by using C2C12 cells. Furthermore, to elucidate the biological significance of TauT/taurine in muscle cells, we tested the

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Taurine 6
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effect of taurine against dexamethasone-induced muscle injury. This provides new insight into the function of TauT/taurine in myogenic differentiation environment.

2. METHODS

2.1 Plasmids

A fragment from -862 to +46 of TauT promoter region were each generated by PCR using primers followed below, and cloned into pGL3 basic Luciferase Vector (Promega, Madison, WI, USA), pTauT/-862-Luc.

-862 (forward):5'-GGGGTACCACACTCCCCATCTCCATTTAAAAATAGTCACA TTTGCTGA-3', +46 (reverse): AAAAGCTTCTAGATGGCACGGGAGTTCA.

2.2 Cell Culture

C2C12 cells were maintained in growth medium (GM), comprising DMEM supplemented with 20% fetal bovine serum, and penicillin (100 U/ml) streptomycin (100 mg/ml) antibiotic. Differentiation of the myoblasts into myotubes was induced by switching the culture medium to the differentiation medium (DM), consisting of DMEM supplemented with 5 μg/ml insulin–transferrin-selenite and penicillin-streptomycin antibiotics**.**

2.3 Northern Blot Analysis

Northern blotting was performed as described previously (Ito *et al*., 2004). In brief, Total RNA was extracted from C2C12 cells at 0 day when cells were cultured in GM and 2 days after differentiation. Total RNA (10 μg) was separated by electrophoresis in 1.5% agarose-formamide gel and was then transferred to a nylon membrane (Hybond N+, Amersham Biosciences, Piscataway, NJ). The membranes were prehybridized in QuickHyb hybridization solution (Stratagene, San Diego, CA, USA) at 68°C for 1 hour, followed by the hybridization with labeled probes at 68°C for 1 hour. The membranes were washed with the buffer containing 2×SSC plus 0.1% SDS and autoradiographed. The intensities of the bands for TauT mRNA were measured by an image analyzer (Fuji Film, Japan) and were normalized to those for GAPDH mRNA.

2.4 Western Blot Analysis

Total protein was collected from C2C12 cells with SDS/PAGE solution. Proteins were separated on a 12.5% polyacrylamide-SDS gel, and then transferred onto Immobilon-P membrane (Millipore). The membrane was probed with TauT specific polyclonal antibody (Alpha Diagnostic International, San Antonio, TX, USA) diluted 1:1000, and the blot was reprobed with GAPDH antibody (Chemicon, CA, USA) as a loading control. Binding was visualized by ECL system.

2.5 Reporter Gene Assay

For luciferase assay, 1×10^4 cells were plated on 12-well plates, cultured in GM overnight and transfected with the TauT/-862-pGL3 plasmids (0.3 μg /well) as described above using FuGene6™ (Roche Applied Science, Indianapolis, IN, USA) transfection agent according to the manufacturer's protocol. For normalization of luciferase activity, the pRL-TK control vector containing the herpes simplex virus thymidine kinase promoter encoding Renilla luciferase (0.2 μg/well) was co-transfected with pGL3 plasmids. Luciferase activity was normalized to that of the Renilla luciferase activity in each experiment. Cells were cultured for 18 h after transfection, the medium was then changed to DM and the cells were cultured further 2 days. Cells were lysed with the Passive Lysis Buffer (Promega). Lysates were analyzed using Dual-Luciferase Reporter Assay System™ kit (Promega).

2.6 Myotube Diameter Measurement

C2C12 cells were cultured in DM for 3 days, and then treated with DM containing control or 100μM dexamethasone (DEX) with or without 2 mM or 20 mM taurine. Different 3 points diameter of myotubes was measured per myotubes. The test was repeated at least 3 times.

3. RESULTS AND DISCUSSION

3.1 Upregulation of TauT During Myogenic Differentiation

C2C12 cells are well-characterized in vitro model of skeletal muscle differentiation. They remain proliferate when they are in mitogen-rich high serum medium (growth medium: GM), but start to differentiate when the medium is switched to serum-restricted medium (differentiation medium: DM) (Yaffe and Saxel, 1977) (Fig. 1A). To determine the regulation of TauT expression during differentiation, TauT expression was studied by Western blot analysis and Northern blot analysis. TauT mRNA was more highly expressed in differentiated myotubes than in proliferating myoblasts (Fig. 1B). In parallel with mRNA expression, TauT protein was upregulated in myotubes (data not shown).

Next, we carried out the luciferase assay by using promoter-reporter construct containing 5'-flanking region proximal 862 bp to transcript start site, pTauT/-862-Luc. Consistent with the upregulation of TauT in myotube, the promoter activity of *TauT* gene was enhanced in C2C12 cells cultured under the differentiation conditions (219% $p<0.01$, vs. GM). These findings indicate that TauT expression is increased at the transcriptional level during muscle differentiation.

Here, a question has arisen if this process is triggered by myogenic differentiation or serum deprivation. Firstly, because the serum inevitably contains taurine which downregulates TauT expression (Jones *et al*., 1991; Shimizu *et al*., 2003), it might be possible that serum depletion (i.e. taurine depletion) results in the induction of TauT. Secondly, because serum deprivation could influence to medium-osmorality, the change

of extracellular osmolality would lead to the upregulation of TauT. However, in our study, treatment with bFGF, which inhibits myogenic differentiation of C2C12 cells, inhibited myotube formation and cancelled the upregulation of TauT mRNA in C2C12 cells (data not shown). Thus, the upregulation of TauT mRNA in differentiated myotube may be independent of the change for extracellular osmolality or taurine concentration but dependent on signal pathway related to myogenic differentiation.

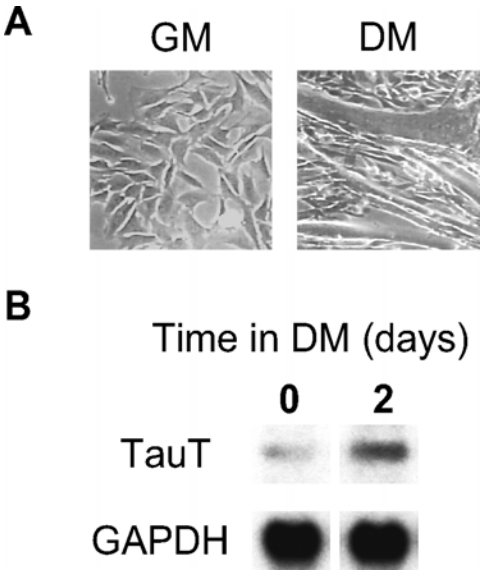

Figure 1. TauT mRNA expression increased during differentiation in C2C12 cells. A. Morphology of C2C12 myoblasts grown in GM (left), and myotubes cultured in DM (right). B. Cells were grown to confluence in GM and then induced to differentiate in DM. mRNA were prepared on the indicated days. The experiment was repeated three times with similar results.

3.3 Anti-Atrophic Effect of Taurine

Skeletal muscle mass and fiber size are thought to be regulated by a dynamic balance of anabolic and catabolic processes. Muscle atrophy is a characteristic response to catabolic state in diverse pathology, such as cancer cachexia, AIDS, diabetis, and chronic obstructive pulmonary disease (Lecker *et al*., 1999).

In this study, to elucidate biological significance of TauT expression in skeletal muscle, we analyzed the effect of taurine on muscle atrophy induced by synthetic glucocorticoid dexamethasone (DEX). After C2C12 cells were cultured in DM for 3days, when they were terminally differentiated, 100 μM DEX was added with or without taurine into the culture medium. While DEX significantly reduced C2C12 muscle cell size, treatment with taurine at 2 mM protected cells from DEX-induced atrophic response, as observed by using a confocal microscope (data not shown). This

observation suggests that an increase in intracellular taurine content through TauT plays an important role in maintenance of normal muscle morphology.

Loss of muscle protein is due predominantly to activation of the ubiquitin–proteasome proteolytic system (Jagoe and Goldberg, 2001). Recently, Foxo transcription factors play a crucial role in muscle atrophy (Sandri *et al*., 2004), and PI-3K/Akt pathway inhibited this atrophy program (Stitt *et al*., 2004). In our previous report, we have demonstrated that taurine treatment suppressed simulated ischemia-induced Akt innactivation in cultured cardiomyocytes and attenuated cell injury (Takatani, 2004), suggesting that taurine may prevent DEX-induced catabolic process via activating Akt-related signal pathway. Further investigation into details is required to clarify the function of taurine/TauT.

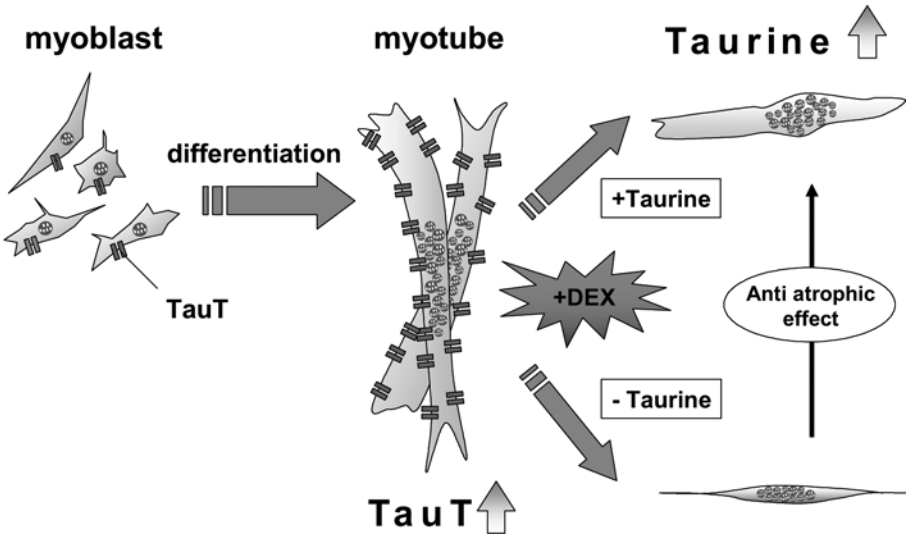

Figure 2. Upregulation of TauT during differentiation and anti-atrophic effect of taurine.

4. CONCLUSION

We demonstrate for the first time that TauT expression is upregulated during differentiation in C2C12 cells. In addition, taurine have a protective effect on muscle atrophy. Thus, taurine may be a therapeutic agent for cachexic state of diverse pathology.

5. ACKNOWLEDGMENTS

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REGIONALLY PERFUSED TAURINE

Part I. Minimizes Lactic Acidosis and Preserves CKMB and Myocardial Contractility after Ischemia/Reperfusion

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1. INTRODUCTION

Taurine attenuates oxidative damage to DNA (Messina and Dawson, 2000). Taurine increases the expression of mitochondria-stabilizing anti-apoptotic Bcl-2 protein while decreasing that of the pro-apoptotic Bax protein and p53 the apoptosis initiator gene (Takahashi *et al.*, 2003), rendering cultured rat cardiomyocytes resistant to hypoxia-induced injury. However, it is not clear whether aerobic or anaerobic mechanisms mediate those anti-apoptotic effects. The hypothesis that taurine preserves aerobic energy and enzyme activities and therefore provides a better function of myocardium subjected to ischemia/reperfusion was now tested in two groups of isolated rat hearts (a) in normothermic ischemia induced while beating, and (b) when high-K^+-depolarized and cold-preserved for 6 hours.

2. MATERIAL AND METHODS

We used adult male Sprague-Dawley rats (350-450 g body weight). All animals received humane care consistent with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institute of Health. The rats were anesthetized with diethylether inhalation supplemented with intraperitoneal sodium pentobarbital (50 mg/kg).

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2.1. Heart Isolation and Perfusion

2.1.1. Thirty-Minute Ischemia (37.5°C) Group

Following laparotomy, heparin (1000 IU/kg) was injected into the inferior vena cava. The heart was exposed via median sternotomy. In the 30-min ischemia (37.5°C) group (A) the hearts were rapidly excised, weighed, and mounted on a non-recirculating Langendorff apparatus. Krebs-Henseleit buffer [KHB without or with 10 mmol/l taurine (Sigma-WAKO, Tokyo, Japan) KHB+T] was perfused by gravity at a constant pressure of 100 mmHg at 37.5°C. KHB composition was NaCl 118 mM, KCl 4.7 mM, $MgSO_4$ 1.2 mM, $NaHCO_3$ 25 mM, KH_2PO_4 1.2 mM, $CaCl_2$ 2.5 mM, and glucose 11 mM, pH 7.4, gassed with a mixture of 95% oxygen and 5% carbon dioxide. After 20-min perfusion stabilization, ischemia was induced while the hearts were beating and maintained for 30 min at ambient temperature.

2.1.2. Six-Hour Hypothermic Preservation Group

The hearts were arrested with an aorta injection of (20 ml/kg) of plain 4°C Saint Thomas Hospital Solution [(Group B) STS, n=8] or containing 10 mmol/l taurine (T) [(Group B) STS+T, n=8], excised, and immediately submerged at 4°C in 40 ml of plain STS or STS containing 10 mmol/l T for 6 hours. The composition of STS was NaCl 110 mM, KCl 16 mM, MgCl 16 mM, $CaCl_2$ 1.2 mM, and $NaHCO_3$ 10 mM).

The hearts in both groups were re-perfused for 20 min on the Langendorff apparatus with plain KHB at 37.5°C and weighed. The pre-ischemic normothermic hearts served as control for the 6-h preservation hearts, *in lieu* of their own pre-arrest data.

2.2. Evaluation of Left Ventricular Function

2.2.1a. Ventricular Pressure. A latex balloon was inserted into the LV cavity through the left atrium. Its end-diastolic pressure was set at 8 mmHg and connected to a pressure transducer for left ventricular pressure (LVP) measurements. Heart rate (HR), LV developed P (LVDP), and the positive maximum rate of P rise (LVP dp/dt, mmHg/s) were recorded at 5 and 20 min of reperfusion in all hearts. In the 30-min ischemic hearts pre-ischemia data were also obtained.

2.2.1b. Coronary Flow. The coronary effluent was collected for one minute. The coronary effluent collected as soon as re-perfused (time 0) was assumed to contain whatever was released during ischemia. The coronary flow index or coronary flow/g heart weight/min (CFi=ml/gHW/min) was calculated using the pre-arrest weight for the pre-arrest, 0-, and 5-min groups to CFi, and the post-20-min reperfusion weight for 20-min CFi.

2.3. Biochemical Analyses

The following components were analyzed in the collected effluents: (a) The energy metabolites pyruvic (PA) and lactic acids (LA) were measured (mg/dl), converted to efflux index (mg/gHW/min) using the proper CFi, and the ratio (LAi/PAi) was calculated. (b) The vital enzymes glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), creatine phosphate kinase (CPK), creatine kinase myocardial

isoform (CKMB), and lactate dehydrogenase (LDH) were measured using an auto-analyzer AU5200 (Olympus, Tokyo, Japan). The enzyme activities were expressed in international units per liter (IU/l) and converted to the enzyme efflux index (IU/gHW/min) using the proper CFi.

2.4. Immunohistochemical Assay of 8-Hydroxy-2'-Deoxyguanosine (8-OHdG)

Only the six-hour preservation hearts were stained for 8-OHdG. After the 20-min collection of functional data and coronary efflux samples, the tissue levels of 8-OHdG were measured as a marker of DNA oxidative stress (Kasai and Nishimura, 1984; Toyokuni, 1999). Cardiac tissue from the ventricular septum was fixed overnight in Bouin's solution and dehydrated sequentially for 24 hours with both 50% and 70% ethanol. The specimen blocks were embedded in paraffin, cut into 3.5 μm thick slices and placed on silane-coated glass slides. The mounted slices were then de-waxed. The avidin-biotin complex method was used for immunohistochemical analysis (Toyokuni *et al.,* 1997), which stains dark the nuclei containing 8-OHdG. The following appropriately diluted solutions were sequentially applied: (a) normal rabbit serum (for inhibition of non-specific binding of the secondary antibody; Dako Japan Co., Ltd, Kyoto, Japan), (b) mouse monoclonal antibody against 8-OHdG (Japan Institute for the Control of Aging, Fukuroi, Japan), (c) biotin-labeled rabbit anti-mouse IgG serum (Dako), and (d) avidin-biotin complex (Vector Laboratories, Burlingame, CA, USA). The substrate for alkaline phosphatase (black) was obtained from a vector. Nuclear counter-staining was not performed.

The level of 8-OHdG was measured over 400X magnification microscopic fields in triplicate slices of each heart. After converting the dark-stained nuclei microscopic images to gray scale (Photoshop version 6.0, Adobe Systems Inc, San Jose, CA, USA), the staining density was quantified as gray scale absorbency using the Scion Image Beta 4.02 (NIH Image version 1.6; Scion Corporation, Frederick, MD, USA) (Toyokuni *et al.,* 1997).

2.5. Statistical Analyses

The commercially available software PRISM version 3.0 (Graph Pad Software Inc, San Diego, CA. USA) was used. All data are expressed as the mean ± standard deviation. Groups were compared using one-way ANOVA followed by Neuman-Keuls test for multiple comparisons. Continuous variables were compared using the paired Student t test and independent variables with the Mann-Whitney test. To assess the taurine effects on the energy metabolites and various enzymes at the different post-reperfusion times and their role relative to LV function, the different parameters were correlated to each other by linear regression. This identified the analyte(s) in which taurine-induced changes, even without significance by Newman-Keuls post-test, affected LV function significantly, and correlated most with other analyte(s) for both heart groups. P value <0.05 was considered significant.

3. RESULTS

3.1. Normothermia (30-min Ischemia)

3.1.1. Functional Effects

KHB+T hearts: Among all measured parameters, the post-reperfusion 5-min LVDP was the only one, which was significantly higher. Post-reperfusion coronary flow indexes were higher at 0 and 5 min but lower at 20 min in both groups compared to pre-ischemic values. T>no-taurine at each reperfusion time but the differences were statistically not significant. Ischemia lasting 30 min induced a weight increase in both groups, though in T<no-taurine hearts, the differences were not significant (Table 1)**.**

Table 1. Physiological data comparison

	Post-reperfusion timing (minutes)	KHB mean±SD	KHB(T) mean±SD
LVDP (mmHg)	Pre	74.22 ± 23.02	79.00 ± 15.56
	5	94.89 ± 11.6 ^{c}	117.5 ± 22.5**, ^{ccc}
	20	69.11 ± 16.5	81.4 ± 16.36
LV dp/dt max (mmHg/s)	Pre	1190 ± 359.9	1234 ± 227.8
	5	1616 ± 308.8 ^{c}	1795 ± 280.7 ^{ccc}
	20	1080 ± 289.1	1261 ± 225.1
CFI (ml/gHW/min)	Pre	10.25 ± 1.39	10.49 ± 1.2
	0	13.18 ± 1.19 ^{cc}	14.63 ± 2.54 ^{ccc}
	5	15.64 ± 1.58 ^{ccc}	16.07 ± 2.21 ^{ccc}
	20	7.45 ± 1.72 ^{cc}	8.30 ± 1.6 ^{c}
Heart rate (HR) (beats/min)	Pre	294.2 ± 24.32	277.8 ± 20.6
	5	298.4 ± 29.46	265.0 ± 37.59
	20	275.8 ± 26.05	245.4 ± 31.04
Heart weight (g)	Pre	1.07 ± 0.07	1.04 ± 0.09
	Post	1.44 ± 0.09 ^{ccc}	1.37 ± 0.16 ^{ccc}

Abbreviations: CFI=coronary flow index; KHB(T)= Krebs Henseleit Buffer (T=taurine); LVDP= left ventricular developed pressure; dp/dt max=rate of change of maximal developed pressure; HB=heart beating; rep=reperfusion; *=p<0.05; **=p<0.01; ***=p<0.001superscripts on the mean±SD are post-Newman Keuls comparing with and without taurine within the group; ^{c,cc,ccc} when compared to pre-ischemia or control.

Although there were some differences between the KHB and KHB+T hearts, none of them were significant. However, characteristically during ischemia (measured in the 0-min coronary effluent) (a) LA is massively produced, KHB (99.12-fold of pre-ischemia)>KHB+T (82.33-fold), (b) PA of KHB+T (3.25-fold of pre-ischemia)>KHB (1.6-fold), (c) CPK of KHB+T (4.7-fold of pre-ischemia)>KHB (2.33-fold of pre-ischemia), and (d) CKMB is not generated (unchanged from pre-ischemia levels) in either group (Table 2, Fig. 1A,B).

3.2. Biochemical Effects

Table 2. Biochemical data

Analyte efflux index	Pre-ischemia No-taurine	Pre-ischemia Taurine	Post-reperfusion time (0 min) No-taurine	Post-reperfusion time (0 min) Taurine
GOT	0.012 ± 0.009	0.004 ± 0.004	0.033 ± 0.044	0.013 ± 0.019
GPT	0.003 ± 0.005	0.003 ± 0.005	0.004 ± 0.008	0.001 ± 0.00
LDH	0.026 ± 0.019	0.027 ± 0.030	0.121 ± 0.187	0.082 ± 0.117
CPK	0.351 ± 0.302	0.153 ± 0.188	0.817 ± 0.931	0.719 ± 1.01
MBCK	0.035 ± 0.011	0.028 ± 0.010	0.034 ± 0.010	0.029 ± 0.012
PA	0.005 ± 0.005	0.004 ± 0.005	**0.008 ± 0.005** ^{cc}	**0.013 ± 0.009 ns** ^{cc}
LA	0.038 ± 0.031	0.040 ± 0.032	3.734 ± 1.074 ^{ccc}	3.293 ± 1.690 ^{ccc}
LA/PA	3.823 ± 3.246	3.801 ± 3.004	451.8 ± 201.4 ^{ccc}	417.0 ± 339.5 ^{ccc}

	Post-reperfusion time 5 min No-taurine	Post-reperfusion time 5 min Taurine	Post-reperfusion time 20 min No-taurine	Post-reperfusion time 20 min Taurine
GOT	0.086 ± 0.056 ^{cc}	0.059 ± 0.068 ^{c}	0.030 ± 0.041	**0.016 ± 0.017**
GPT	0.016 ± 0.002 ^{ccc}	0.020 ± 0.014 ^{ccc}	0.009 ± 0.004	0.009 ± 0.002
LDH	0.189 ± 0.130 ^{c}	0.168 ± 0.139	0.072 ± 0.112	**0.043 ± 0.031**
CPK	0.647 ± 0.525	0.510 ± 0.532	0.236 ± 0.435	**0.099 ± 0.139**
MBCK	0.092 ± 0.018 ^{ccc}	0.084 ± 0.018 ^{ccc}	0.043 ± 0.013	0.043 ± 0.014
PA	0.017 ± 0.006	0.016 ± 0.004 ^{ccc}	0.007 ± 0.005	0.007 ± 0.002
LA	**0.106 ± 0.078**	**0.083 ± 0.048 ns**	**0.057 ± 0.049**	**0.038 ± 0.015 ns**
LA/PA	**6.460 ± 4.109**	**6.015 ± 5.719 ns**	**9.390 ± 4.981**	**5.985 ± 4.525 ns**

Means±SD shown. Abbreviations: efflux index for enzymes=IU/gHW/min, energy metabolites=mg/gHW/min, HW=heart weight, min=minute, CKMB=creatine phosphokinase myocardial isoform, CPK=creatine phosphokinase, GOT=glutamate oxaloacetate transaminase, GPT=glutamate pyruvate transaminase, LDH=lactate dehydrogenase, LA=lactic acid, and PA=pyruvic acid. Statistical significance: *=p<0.05; ***=p<0.001; ****=p<0.0001; taurine *vs.* no-taurine; ^{c}=p<0.05 *vs*. control.

Table 3A. Correlation of 0-min PA, 5-min LA, and 5-min CKMB to functional parameters

(A)	0-min r^2	0-min P value	0-min DIR	5-min LA r^2	5-min LA P value	5-min LA DIR	5-min CKMB r^2	5-min CKMB P value	5-min CKMB DIR
LV dp/dt 5-min (all)				0.3332	**0.015**	-			
Cfi 5-min (all)							0.2379	**0.034**	+
Cfi 20-min Tau							0.379	0.0581	+

For abbreviations see Tables 1 and 2. DIR=direction of the linear regression line.

3.3. Linear Correlation

To determine which of the taurine effects played a role in determining the 5-min LVDP or 5-min LVdp/dt, the various parameters were correlated by linear regression (Table 3). Taurine effects are apparent as early as during ischemia evidenced as promoting the production of 0-min PA and abating that of 0-min LA, thus decreasing the 0-min LAi/PAi ratio. Albeit the differences were not significant (Table 2), the correlation was highly significant, especially in decreasing LA during ischemia (Tables 3A,B, Fig. 2A,B). The 5-min LA correlated most often with 5-min parameters (positively for LA/PA ratio, but negatively for CKMB and LVdp/dt) and with 20-min parameters (positively for

CPK, GOT and LDH). The 5-min CKMB correlated positively with 5-min CFi, 5-min GPT, pre-ischemia, 0-min and 20-min CKMB; but negatively with 0-min and 5-min LA.

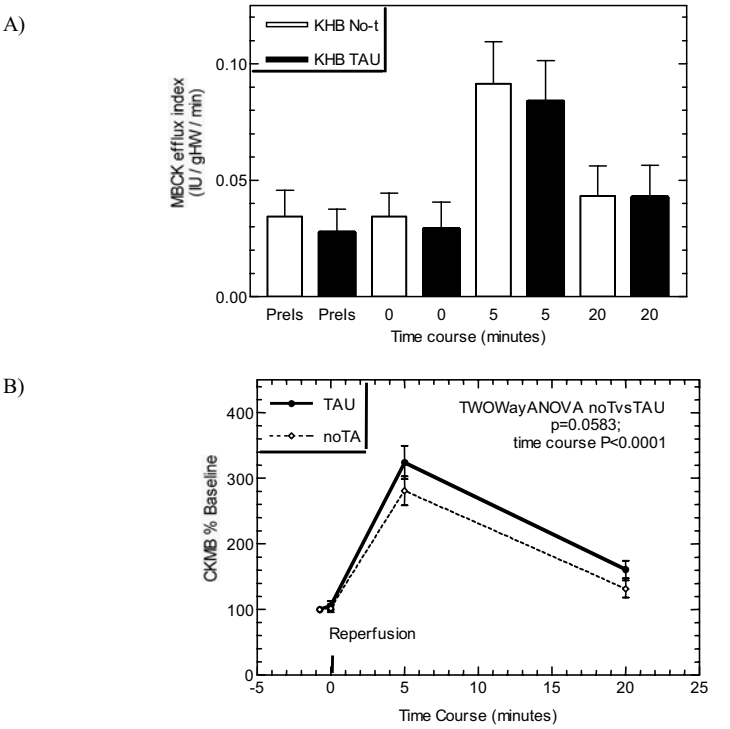


Figure 1. (A) CKMB is not generated during ischemia, regardless of the presence or absence of taurine but (B) more CKMB is generated post-reperfusion by KHBT than KHB no-T hearts relative to the baseline levels.

4. DISCUSSION

This model examines the direct effects of taurine on the target organ independent from the effects, which could be obtained by systemic administration. It corroborates the established fact that as soon as ischemia occurs, the energy metabolism becomes anaerobic with massive production of lactic acid. Furthermore, it demonstrates that CKMB production ceases (time 0-min = baseline levels), though that of CPK increases slightly. Contrary to the widespread concept of equating CKMB to injury, it is evident that CKMB, an absolutely O_2-dependent enzyme, is the main stem of the aerobic machinery in the heart. A higher 5-min CKMB resulting from the leaky membrane that normalizes after 20 min is not yet necessarily an injury marker at this time. Although taurine did not support CKMB production during ischemia, it supported other aerobic pathways. Pyruvic acid then leads to the recovery of normalcy of all enzymatic activities (GPT, GOT, CPK, LDH, CKMB) faster than in KHB hearts after reperfusion. The fact that KHB+T hearts produced more pyruvic and less lactic acid during ischemia and initial 5-min reperfusion than KHB hearts, even though the differences were not significant, is

meaningful when analyzed by linear regression. The 5-min lactic acid correlates negatively with LV function and CKMB efflux at 5 min, and positively with the GOT, LDH and CPK at 20 min. It indicates that 5-min lactic acid depresses early CKMB production necessary for oxidative phosphorylation and LV function, and increases the efflux of other injury enzymatic markers at 20 min. The release of CKMB at 5 min post-reperfusion is thus not an indication of injury but actually represents ability for aerobic metabolism.

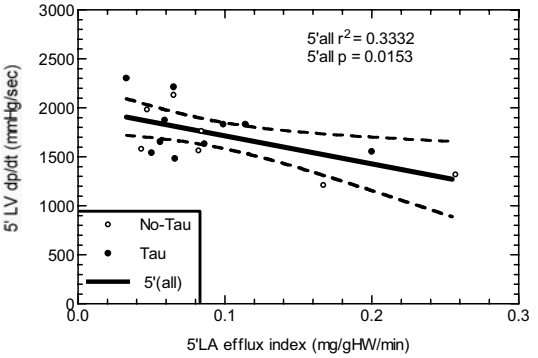
Although no pathological studies or TUNEL staining were performed, it is evident that taurine supported aerobic metabolism during 30-min ischemia which minimized lactic acidosis and its consequences on Bcl and Bad expressions (Thatte *et al.,* 2004). This prevented apoptosis and allowed LVP to recover 5 minutes after reperfusion, significantly faster than the hearts without taurine.

Table 3B. Correlation of 0-min PA, 5-min LA, and 5-min CKMB to biochemical parameters

(B)	0-min PA		DIR	5-min LA		DIR	5-min CKMB		DIR
	r^2	P value		r^2	P value		r^2	P value	
0-min GOT No-Tau	0.6605	**0.0263**	+						
20-min GOT Tau				0.4194	**0.043**	+			
20-min GOT (all)				0.4053	**0.006**	+			
5-min GPT Tau							0.5673	**0.012**	+
5-min GPT (all)							0.2549	**0.028**	+
PreLDH No-T	0.8339	**0.0041**	+						
0-min LDH No-Tau	0.6739	**0.0236**	+						
20-min LDH (all)				0.2572	**0.038**	+			
20-min CPK Tau				0.5439	**0.015**	+			
20-min CPK (all)				0.4085	**0.006**	+			
PreMBCK (all)							0.4357	**0.002**	+
0-minCKMB No-Tau							0.7263	**0.004**	+
0-min CKMB Tau							0.4065	**0.047**	+
0-min CKMB (all)							0.5544	**0.0003**	+
5-min CKMB No-Tau				0.5705	**0.05**	-			
5-min CKMB (all)				0.2358	**0.048**	-			
20-min CKMB Tau							0.5833	**0.010**	+
20-min CKMB (all)							0.3114	**0.013**	+
0-min LA No-Tau							0.8575	**0.003**	-
0-min LA Tau	0.4639	**0.0301**	-						
0-min LA (all)	0.3512	**0.0122**	-						
5-min LA No-Tau							0.5705	**0.050**	-
5-min LA (all)							0.2358	**0.048**	-
0-min L/P Tau	0.5962	**0.0089**	-						
0-min L/P (all)	0.5709	**0.0007**	-						
5-min L/P No-Tau				0.7804	**0.008**	+			
5-min L/P Tau				0.9037	**< 0.0001**	+			
5-min (L/P) (all)				0.7057	**< 0.0001**	+			

For abbreviations see Tables 1 and 2. DIR=direction of the linear regression line. A greater number of parameters correlate with 5-min lactic acid.

A)



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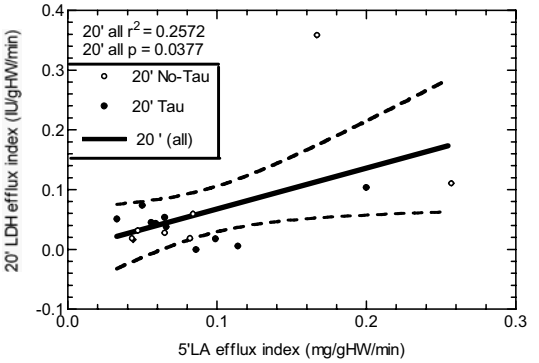

Figure 2 A. Five-min LA correlates negatively with 5-min LV dp/dt, and (B) positively with 20-min LDH.

4.1. Limitations and Significance of the Study

The actual temperature in the hearts was not measured and the hearts were maintained at room temperature. Nevertheless, it seems unquestionable that the release of CKMB by itself during early post-reperfusion is not an indication of injury in this model.

Part II. Taurine Addition to St Thomas' Solution Prevents DNA Oxidative Stress and Maintains Contractile Function

1. INTRODUCTION

Cardioplegia reduces cellular energy expenditure and severity of myocardial ischemia/reperfusion injury, but eventual damage is ischemia-time dependent secondary to Ca^{2+} overload and its effects on reactive oxygen species (Miyamae *et al.,* 1996; Sharikabad *et al.,* 2000). The acidosis-induced by ischemia activates 4 pro-apoptotic factors, which impairs myocardial ventricular function (Thatte *et al.,* 2004). DNA oxidative stress generates 8-hydroxy-2'-deoxyguanosine (8-OHdG), which correlates with the severity of apoptosis and functional impairment (Yamazaki *et al.,* 2004). Taurine minimized lactic acid production during short ischemia. We sought to evaluate whether taurine protection involved acidosis and DNA oxidative stress prevention in the hearts arrested by high-K^+ depolarization and cold-preserved for 6 hours.

2. MATERIAL AND METHODS

These were described for the normothermic hearts in part I. The only difference is that 0-min coronary effluents were not collected. The LV pressure at 5 minutes could not be assessed in most hearts. The time required for the heartbeat to stabilize was measured instead. Pre-ischemia data of KHB hearts were used as control.

3. RESULTS

3.1. Functional Effects

All parameters were significantly different between the Saint Thomas cardioplegic solution (STS)+taurine (T) and the plain STS. The heartbeat was stabilized faster, the weight increase smaller, and the 5- and 20-min CFi, 20-min LVDP, and 20-min LVdp/dt were markedly greater (Table 4).

3.2. Biochemical Effects

The significantly greater amounts of 5-min PA, 5- and 20-min CKMB, and 5-min GPT; and the smaller 20-min LAi/PAi ratio of STS+T compared to STS hearts are notorious. The two most outstanding differences were the greater amount of 5-min CKMB and the complete prevention of 8-OHdG staining (Table 5, Fig. 3A,B).

Table 4. Physiological data comparison

	Post-rep time (minutes)	STS mean±SD	STS+T mean±SD	Control hearts mean±SD
LVDP (mmHg)	20	70.63 ± 21.19 ^{ccc}	99.75 ± 14.18**	119.2 ± 15.45
LV dp/dt max	20	1031 ± 306.1 ^{ccc}	1566 ± 181***, ^{cc}	1917 ± 127.4
CFI	5	12.28 ± 2.299	17.73 ± 2.111***	
	20	7.231 ± 1.984 ^{ccc}	10.84 ± 1.515***	12.99 ± 2.21
HR	20 min	272 ± 34.11	286 ± 33.5	271.6 ± 22.53
HW	Pre-ischemia	1.08 ± 0.058	1.056 ± 0.081	1.132 ± 0.160
	Post 20-min rep	1.615 ± 0.057 ^{ccc}	1.543 ± 0.104*, ^{ccc}	
Time to stable HB (s)	Post-rep	472.1 ± 102.1	248.8 ± 71.8***	

Abbreviations: CFI=coronary flow index, KHB(T)= Krebs-Henseleit buffer (T=taurine), LVDP=left ventricular developed pressure, dp/dt max=rate of change of maximal developed pressure, HB=heart beating, and rep=reperfusion. *=p<0.05; **=p<0.01; ***=p<0.001superscripts are post-Newman Keuls comparing no-taurine vs. taurine within the group; ^{c,cc,ccc} are *vs.* control.

Table 5. Biochemical data

	Control	**Post-reperfusion time 5 min**	
		No-taurine	**Taurine**
GOT	0.027 ± 0.021	1.554 ± 0.653	1.755 ± 0.523
GPT	0.028 ± 0.027	**0.082 ± 0.030**	**0.148 ± 0.115***
LDH	0.063 ± 0.037	3.915 ± 1.901	4.066 ± 1.26
CPK	0.063 ± 0.053	14.01 ± 5.962	15.37 ± 4.491
CKMB	0.078 ± 0.014	**0.073 ± 0.010**	**0.106 ± 0.014*****
PA	0.008 ± 0.004	**0.011 ± 0.006**	**0.02 ± 0.013***
LA	0.055 ± 0.026	0.096 ± 0.080	0.053 ± 0.016
LA/PA	7.336 ± 2.977	**10.7 ± 13.59**	**3.79 ± 2.78**
8-OHdG			

	Control	**Post-reperfusion time 20 min**	
GOT	0.027 ± 0.021	0.401 ± 0.438	**0.147 ± 0.096**
GPT	0.028 ± 0.027	**0.056 ± 0.032**	**0.042 ± 0.038**
LDH	0.063 ± 0.037	0.894 ± 1.095	**0.311 ± 0.191**
CPK	0.063 ± 0.053	3.542 ± 3.673	**1.332 ± 0.961**
CKMB	0.078 ± 0.014	**0.045 ± 0.010**	**0.068 ± 0.013***,^{c}**
PA	0.008 ± 0.004	**0.009 ± 0.004**	**0.009 ± 0.003**
LA	0.055 ± 0.026	0.120 ± 0.150	**0.043 ± 0.026**
LA/PA	7.336 ± 2.977	**11.68 ± 8.09** ^{c}	**4.613 ± 1.723***
8-OHdG		**6386 ± 570**	**3801 ± 529********

Means±SD shown. Abbreviations: efflux index for enzymes=IU/gHW/min, energy metabolites=mg/gHW/min, HW= heart weight, min=minute, CKMB=creatine phosphokinase myocardial isoform, CPK=creatine phosphokinase, GOT=glutamate oxaloacetate transaminase, GPT=glutamate pyruvate transaminase, LDH=lactate dehydrogenase, LA=lactic acid, PA=pyruvic acid, 8-OHdG=8-hydroxy-2'-deoxyguanosine measured as Gray Scale Absorbency. Statistical significance: *=p<0.05; ***=p<0.001; ****=p<0.0001; taurine vs. no-taurine; ^{c}=p<0.05 *vs*. control.

Table 6A. 8-OHdG and 5-min CKMB correlation to functional parameters

	8-OHdG			5-min CKMB		
	r^2	P value	Direction	r^2	P value	
5-min LVDP (all)	0.1549	0.0056	-			
20-min LVDP No-Tau			+	0.6452	< 0.0001	
20-min LVDP (all)	0.3821	< 0.0001	-	+	0.3644	< 0.0001
20-min dp/dt No-T			+	0.6953	< 0.0001	
20-min dp/dt (all)	0.5281	< 0.0001	-	+	0.5212	< 0.0001
CFi 5-min No-Tau			+	0.7884	< 0.0001	
CFi 5-min (all)	0.6155	< 0.0001	-	+	0.678	< 0.0001
CFi 20-min No-Tau			+	0.5554	0.0001	
CFi 20-min (all)	0.4692	< 0.0001	-	+	0.415	< 0.0001
20-min HW (all)	0.1589	0.005	+	-	0.1722	0.0046
Stable beat (all)	0.5471	< 0.0001	+	-	0.5082	< 0.0001

For abbreviations: See tables 1, 2 and 3. More parameters correlate with 5-min CKMB than with 8-OHdG.

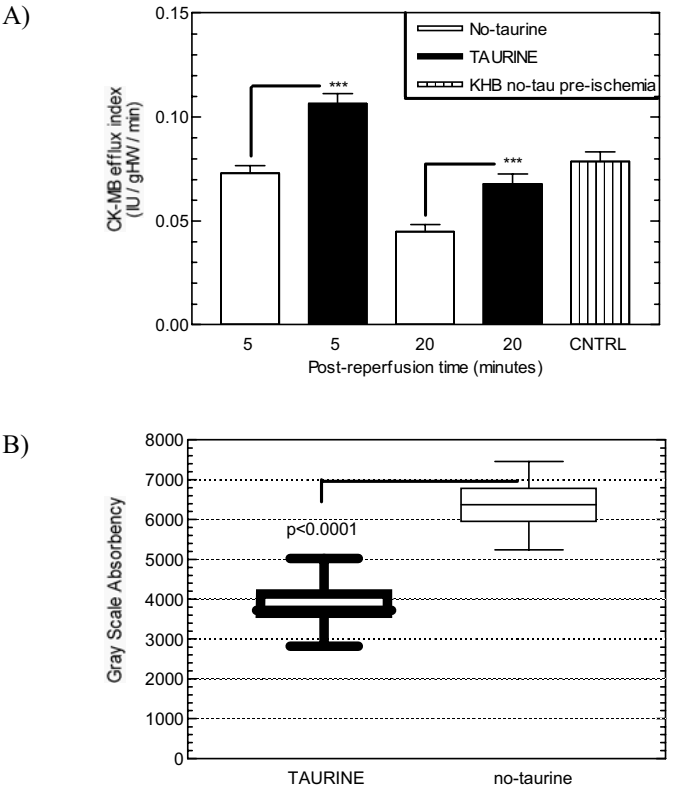

Figure 3. The major differences were (A) taurine hearts released greater amounts of CKMB either at 5 or 20 minutes of reperfusion and (B) taurine prevented DNA oxidative stress in all hearts.

3.3. Linear Correlation

The 20-min 8-OHdG correlated either positively or negatively with all studied parameters, the strongest correlation was with the 5-min CKMB. The 5-min CKMB correlates **POSITIVELY** with (i) 5-min PA, 5- and 20-min GPT; and (ii) 20-min CKMB and ventricular functions (Fig. 5), and **NEGATIVELY** with (i) 5- and 20-min LA and LA/PA, (ii) 20-min GOT, LDH and CPK, (iii) post-reperfusion beating stabilization time, and (iv) HW at 20 min (Tables 6A,B, Figs. 4A B).

Table 6B. 8-OHdG and 5-min CKMB correlation to biochemical parameters

	8-OHdG		5-min CKMB			
	r^2	P value	Direction		r^2	P value
5-min CKMB No-Tau				na	1	< 0.0001
5-min CKMB Tau				na	1	< 0.0001
5-min CKMB (all)	**0.6612**	**< 0.0001**	-	na	1	< 0.0001
20-min CKMB No-Tau				+	0.4013	0.002
20-min CKMB (all)	0.4653	< 0.0001	-	+	0.5503	< 0.0001
5-min GPT Tau				+	0.3369	0.0029
5-min GPT (all)	0.1408	0.0111	-	+	0.2834	0.0002
20-min GPT Tau				+	0.3175	0.0041
20-min GOT No-Tau				-	0.2029	0.0404
20-min GOT (all)	0.125	0.0137	+	-	0.2455	0.0005
20-min LDH (all)	0.1068	0.0234	+	-	0.2385	0.0007
20-min CPK (all)	0.1306	0.0116	+	-	0.2719	0.0002
5-min LAi No-Tau				-	0.3003	0.0101
5-min LAi (all)	0.1219	0.0187	+	-	0.2458	0.0005
20-min LAi No-Tau				-	0.224	0.0302
20-min LAi (all)	0.1181	0.0168	+	-	0.1749	0.0043
5-min PAi No-Tau				-	0.2029	0.0404
5-min PAi (all)	0.1665	0.0054	-	+	0.1327	0.0139
5-min LAi/PAi Tau				-	0.2858	0.0071
5-min LAi/PAi (all)	0.0995	0.029	+	-	0.2185	0.0012
20-min LAi/PAi No-Tau				-	0.4391	0.0011
20-min LAi/PAi (all)	0.2573	0.0002	+	-	0.3238	< 0.0001

For abbreviations see Tables 1, 2, and 3. More parameters correlate with 5'CKMB than with 8-OHdG.

4. DISCUSSION

Despite the weight increase of all hearts, both normo- and hypothermic, the increase in T<no-taurine with a better function suggests edema and tissue injury being curtailed by taurine under both conditions (Tables 2, 3, 5, and 6).

Although taurine was present in this study only in the arrest-inducing cardioplegic STS solution but not in the KHB used for reperfusion, it (a) completely prevented the generation of 8-OHdG (Fig. 3B), (b) induced significantly higher CKMB (Fig. 3A), pyruvic acid and GPT at 5-min reperfusion, and not significantly but less lactic acid (Table 5), (c) improved contractile functions at 20-min reperfusion (Table 4; Fig. 5), and (d) reduced 20-min LDH, suggesting less tissue injury (Fig. 4B).

Whether the effect of taurine is mainly on pyruvic acid production and GPT, and secondarily on the CKMB, or the reverse, is only a speculative matter. In part I, taurine supported pyruvic acid production in normothermic ischemia. If the same occurs during

hypothermia, the preservation of pyruvic acid production might be the major mechanism which taurine preserves. The levels of 8-OHdG negatively correlating with 5-min and 20-min CKMB, and strongest with 5-min CKMB (r^2 = 0.66120), and CKMB correlating with GPT and pyruvic acid suggest the major role which CKMB plays on preservation of DNA. Whether or not p53, the apoptosis initiator gene (Takahashi *et al.,* 2003), is linked to CKMB is unknown, but it might be the gene whose oxidation is preventable with taurine. Only the several-hour post-ischemia/reperfusion high CKMB levels seem to represent irreversibly injured and permanently leaky cell membrane.

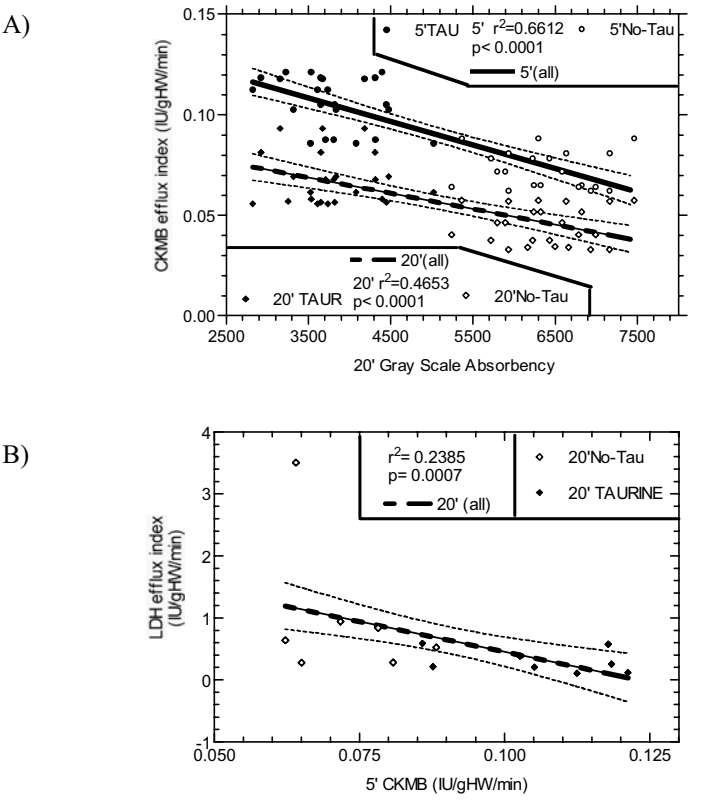

Figure 4. (A) The greater the amount of 5-min CKMB release, the less the amount of oxidized DNA. (B) The greater the amount of 5-min CKMB release, the less the amount of LDH.

The facts that hearts with high levels of 5-min CKMB activity had better 20-min LV function and lesser efflux of LDH indicate less damage, even though pathological studies were not performed. Thus 5-> 20-min CKMB levels most likely indicate a progressive decrease of the leaky membrane at this early time. The higher 5-min CKMB represents the higher ability to support aerobic metabolism, that is, viability but not irreversible injury in this model. Furthermore, the greater correlation of 5-min CKMB with some parameters (GPT, GOT, LDH, CPK, LA, PA, LA/PA and HW) than that of 8-OHdG is

most interesting (Table 6). It suggests that CKMB could be used as an effective surrogate of 8-OHdG at 20 min and/or other apoptotic markers. The aerobic metabolic environment influences most CKMB and GPT (Table 5) and secondarily all other enzymes (Table 6). Though the 20-min CKMB of T>no-taurine hearts, it is still lower than that in control hearts (Table 5) and may explain the significantly lower LVDP and dp/dt compared to control hearts (Table 4) as the highly significant correlation indicates (Fig. 5B). Thus 5- and 20-min CKMB are the determinants of LV function rather than representing injury in this model.

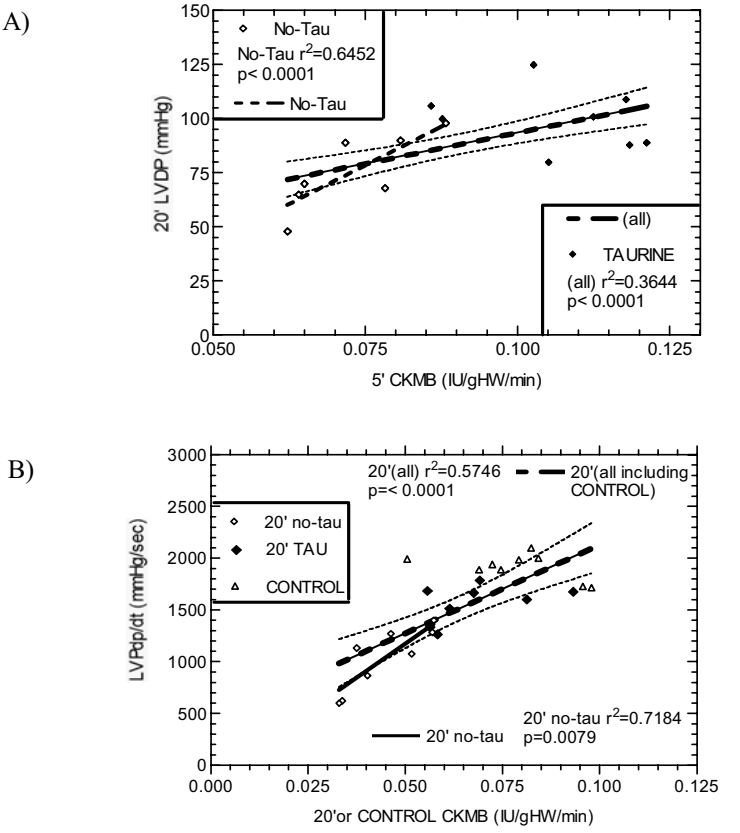

Figure 5. (A) Five-min CKMB correlates positively with the eventual left ventricular function and (B) 20-min CKMB correlates with LV dp/dt. Control hearts without ischemia with the higher release of CKMB generate the greatest LV dp/dt.

The T hearts leaked non-significantly less CPK, GOT, GPT and LDH at 20 min than the no-taurine hearts (Table 5), but still more than the non-ischemic control hearts, suggesting some degree of tissue injury or greater membrane permeability. However, the correlation of 8-OHdG, CKMB, and LA (not shown) with those tissue injury markers indicates less injury than in the no-taurine hearts (Table 6, Fig. 4B). In all reported

experimental studies a better viability is associated with taurine use. Taurine preserves intracellular ATP levels in cultured cardiomyocytes (Messina and Dawson, 2000) or renal cells (Wingenfeld *et al.,* 1996) rendered hypoxic and in hepatocytes exposed to toxins (hydrazine or 1,4-naphtoquinone; Timbrell *et al.,* 1995). Taurine also decreases enzyme leakage from the livers subjected to re-oxygenation after the 16-h ischemic hypothermic storage (Wettstein and Haussinger, 2000).

Ischemia reduces the activity of cellular defense enzymes against free radicals. Reperfusion or re-oxygenation further disturbs the delicate balance of oxidants and antioxidants. The burst of reactive oxygen species (McCord, 1988; Miyamae *et al.,* 1996; Sharikabad *et al.,* 2000) damages DNA (Siesjö *et al.,* 1996; Li *et al.,* 1997, 1999a, 1999b, 2001; Messina and Dawson, 2000; Takahashi *et al.,* 2003). DNA oxidation results in the production of 8-OHdG that can be immunohistochemically identified and quantified (Kasai and, 1984; Toyokuni, 1999). The 8-OHdG levels increase in cardiomyocytes after myocardial infarction (Miwa *et al.,* 2002). Myocardial reperfusion injury could be prevented by inhibiting the poly(ADP-ribose) synthetase, which increases the levels of NAD^+ and decreases the levels of 8-OHdG and apoptosis as assayed by TUNEL (Yamazaki *et al.,* 2004). We used thus 8-OHdG, CKMB, PA, and LA as surrogates of TUNEL and NAD^+ assays.

Thatte *et al*. (2004) reported that the severity of myocardial acidosis determined the extent of activation of 4 pro-apoptotic markers and LV function depression. This study demonstrates beyond any question that taurine prevents 8-OHdG generation, that is, DNA oxidative stress in the cold-preserved heart, but does not address as to which gene's oxidation is affected and how, whether directly on DNA or indirectly via CKMB-catalyzed oxidative phosphorylation or other vital enzymes. However, the fact that taurine ameliorated ventricular function mainly by preserving CKMB activation restoring aerobic metabolism as early as 5 min after reperfusion, minimizing lactic acidosis and preventing DNA oxidation, seems unquestionable even in absence of TUNEL studies. It might be worth mentioning that subsequent TUNEL staining of the studied hearts corroborated the significantly greater number of apoptotic nuclei in 8-OHdG positive cells (Oriyanhan *et al.*, 2005). We assume that these effects are mediated by (a) the increased protein expression of the mitochondria-stabilizing anti-apoptotic Bcl-2 and (b) decreased expression of the pro-apoptotic Bax, as well as p53, the apoptosis initiator gene (Takahashi *et al.,* 2003). It was recently found that taurine inhibition of apoptosis involves Akt-mediated caspase-9 inactivation in cultured cardiomyocytes exposed to hypoxia (Takatani *et al.,* 2004).

Although a significantly higher pyruvic acid efflux was observed only in the 6-h preservation T hearts, taurine decreased lactic acidosis and restored better ventricular function faster post reperfusion in both normothermic and hypothermic hearts. It may thus have a place in protecting hearts during percutaneous intravascular interventions or during off-pump and on-pump cardiac surgery. The relative protective role of taurine in the hypothermic 6-h preserved hearts being far greater than in normothermic short ischemia hearts suggests that it is most effective when the ischemia/reperfusion-induced stress is greatest and most needed, that is, for transplantation.

As described in the previous chapter taurine has significant central sympatholytic effects (Paakkari *et al.,* 1982; Kontro and Oja, 1990; Liljequist, 1993; Avanzino *et al.,* 1994; Ye *et al.,* 1997; Saransaari and Oja, 2000; Kakee *et al.,* 2001; Yoshida *et al.,* 2002 Dampney *et al.,* 2003). Thus, we postulate that administering taurine systemically (Milei *et al.,* 1992; Timbrell *et al.,* 1995; Ohno *et al.,* 1999) to the donor 30~60 minutes before

harvesting might prevent the brain-death-induced cardiotoxicity of endogenous catecholamines (Farhat *et al.,* 2001; Yeh *et al.,* 2002). Addition to STS or any preservation solution (Diodato *et al.,* 2004) should maximize taurine's protective effects. Furthermore, administration of taurine to the recipient before re-establishing flow to the transplanted organ, since it is a most effective anti-oxidant free radical scavenger (Franconi *et al.,* 1985; Wright *et al.,* 1986; Huxtable, 1992; Bkaily *et al.,* 1997; Satoh and Sperelakis, 1998; Schaffer *et al.,* 2002), may further minimize reperfusion injury.

4.1. Limitations and Significance of the Study

Because ATP, CP NAD^+ were not measured, the inferences are based on indirect but widely accepted evidences of aerobiosis. Studies on blood-perfused models of larger animals might be needed to evaluate the detrimental effects of leukocytes, but because we have only amplified the natural taurine defense mechanisms operational in all animal kingdom organs against ischemia/reperfusion injury, similar results could be anticipated. Even if not all mechanisms are elucidated, a better preservation of the harvested donor hearts might increase the donor pool size and will certainly benefit the recipient.

5. CONCLUSIONS

The taurine-perfused isolated rat hearts recovered faster after normothermic 30-min ischemia induced while the hearts were beating. Lactic acid production was decreased and pyruvic acid production increased. The addition of taurine to St. Thomas Hospital cardioplegic solution decreased lactate and efflux of tissue injury markers, maintained CKMB activity and aerobic metabolism, and prevented DNA oxidation, markedly ameliorating LV function in the hearts stored cold for 6 hours

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Part 5.
Taurine in Brain and Retina

THE EFFECT OF OXIDATIVE STRESS ON THE TRANSPORT OF TAURINE IN AN *IN VITRO* MODEL OF THE BLOOD-BRAIN BARRIER

Young-Sook Kang

1. INTRODUCTION

Taurine (2-aminoethanesulfonic acid) is one of the abundant free sulfur-containing beta-amino acids in the central nervous system (CNS) and thought to play a role as a neuromodulator (Oja and Saransaari, 1996). Taurine is also known to exert neuroprotective effects against excitotoxic agents (French *et al.,* 1986) and oxidative stress (Boldyrev *et al.,* 1999). The taurine level in the brain interstitial fluid is elevated in ischemia (Matsumoto *et al.,* 1996; Nakane *et al.,* 1998), indicating that the brain controls its taurine level for protecting neurons in response to cell damage.

The release of taurine from neuronal cells could be one of the regulating mechanisms. The release of taurine from hippocampal slices is increased under hypoglycemic and ischemic conditions, and in the presence of 2,4-dinitrophenol or media inducing free radical production by H_2O_2 (Saransaari and Oja, 2000). Another regulatory mechanism could involve the transport system at the blood-brain barrier (BBB). The BBB, which is formed by a complex of tight junctions of brain capillary endothelial cells, possesses a transport system for amino acids including taurine. The blood-to-brain influx of taurine has been demonstrated by the brain perfusion method (Benrabh *et al.,* 1995). A study using primary cultured bovine brain capillary endothelial cells (BCEC) has shown that there is a taurine transport system in both the luminal and abluminal membranes of the BCEC (Tamai *et al.,* 1995; Tsuji and Tamai, 1996). Therefore, this taurine transport system at the BBB may be involved in the maintenance of taurine levels in the brain.

In our previous study, the transport activity of taurine at the BBB was reduced in spontaneously hypertensive rats in comparison with normotensive rats (Kang, 2000). This result suggests that taurine transport activity is regulated at the BBB, and this regulation is responsive to factors affecting the BBB, such as stress and cell damage, for controlling the neuroprotective effects of taurine. This hypothesis suggests a novel function of the BBB, which controls transport to protect neurons from damage. However, the mechanism

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Taurine 6
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of taurine transport regulation at the BBB, especially when CNS cells are damaged, is unknown.

The purpose of this study is to clarify the regulation of taurine transport using conditionally immortalized rat brain capillary endothelial cell lines (TR-BBB13) as an *in vitro* BBB model (Terasaki and Hosoya, 2001). Furthermore, the expression of taurine transporter (TAUT) in TR-BBB13 cells was investigated to clarify its regulation mechanism.

2. MATERIALS AND METHODS

2.1 Reagents

[2-^{3}H(N)]Taurine ([^{3}H]taurine, 1.12 PBq/mol) and [carboxyl-^{14}C]inulin ([^{14}C]inulin, 0.071 GBq/g) were purchased from NEN Life Sciences, (Boston, MA). Tumor necrosis factor alpha (TNF-alpha, human recombinant (*E. coli*) solution, sterile, 10 μg/ml) was purchased from Boehringer Mannheim Biochemica (Manheim, Germany) and lipopolysaccharide (LPS) from *Salmonella Minnesota R595* (Re) was obtained from List Biological Laboratories (Campbell, CA). Diethylmaleate (DEM) and taurine were purchased from Wako Pure Chemical (Osaka, Japan). Rabbit anti-taurine transporter antibody (TAU11) was purchased from Alpha Diagnostic International (San Antonio, TX). All other chemicals were of reagent grade and available commercially.

2.2 Cell Culture

TR-BBB13 cells (passage number 18-23) were grown routinely in collagen type-I coated tissue culture dishes (Becton Dickinson, Bedford, MA) at 33°C under 5% CO_2 and 95% air as described previously (Hosoya *et al*., 2000). The cells were cultured in Dulbecco's modified Eagle medium (Nissui, Tokyo, Japan) supplemented with 100 U/ml benzylpenicillin (Wako Pure Chemicals, Osaka, Japan), 100 μg/ml streptomycin sulfate (Wako Pure Chemicals), 10% fetal bovine serum (Moregate, Bulimbra, Australia), and 15 μg/l endothelial cell growth factor (Boehringer Mannheim, Mannheim, Germany).

2.3 [^{3}H]Taurine Uptake Study by Cultured TR-BBB13 Cells

[^{3}H]Taurine uptake was assayed according to the previous report of Hosoya *et al*. (2000). TR-BBB13 cells ($1x10^{5}$ cells/well) were cultured at 33°C for 2 days on rat tail collagen type-I coated 24-well plates (Becton Dickinson) and washed with 1 ml extracellular fluid (ECF) buffer consisting of 122 mM NaCl, 25 mM $NaHCO_3$, 3 mM KCl, 1.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.4 mM K_2HPO_4, 10 mM D-glucose, and 10 mM Hepes (pH 7.4) at 37°C. Uptake was initiated by applying 200 μl ECF buffer containing 37 Bq [^{3}H]taurine and 7.4 Bq [^{14}C]inulin as a correction for water adhesion at 37°C in the presence or absence of inhibitors. After appropriate time periods, the uptake was terminated by removing the applied solution and the cells were immersed in ice-cold ECF buffer. The cells were then solubilized in 750 μl 1 M NaOH. An aliquot (50 μl) was taken for protein assay using a DC protein assay kit (Bio-rad, Hercules, CA) with bovine serum albumin as a standard. The remaining solution (500 μl) was mixed with 5 ml scintillation cocktail (Hionic-fluor, Packard, Meriden, CT) for measurement of radioactivity in a liquid scintillation counter (LS6500, Beckman, Fullerton, CA).

2.4 RNA Extraction and Quantitative Real-Time PCR

Total mRNA was extracted from TR-BBB13 cells using an RNA-Plus extraction kit (Q-Bio gene, Illkirch, France) according to the manufacturer's protocol. RNA integrity was checked by electrophoresis on an agarose gel. Quantitative real-time PCR was performed using an ABI PRISM 7700 sequence detector system (PE Applied Biosystems, Foster City, CA) with 2 X SYBR Green PCR Master Mix (PE Applied Biosystems) as per the manufacturer's protocol. To quantify the amount of specific mRNA in the samples, a standard curve was generated for each run using the plasmid (pGEM-T Easy Vector, Promega) containing the TAUT gene or beta-actin (dilution ranging from 0.1 fg/μl to 1 ng/μl). This enabled standardization of the initial mRNA content of TR-BBB13 cells relative to the amount of beta-actin. The PCR was performed using TAUT-specific primers through 40 cycles of 95°C for 30 s, 60°C for 1 min, and 72°C for 1 min after pre-incubation at 95°C for 10 min. The TAUT-specific primers are given above.

2.5 Data Analysis

Unless otherwise indicated, all data are given as mean values ± S.E.M.. An unpaired, two-tailed Student's *t*-test was used to determine the significance of differences between two group means. Statistical significance of differences among means of several groups was determined by one-way analysis of variance (ANOVA) followed by the modified Fisher's least squares difference method, and $p<0.05$ was considered statistically significant.

3. RESULTS

3.1 Effect of TNF-α, LPS and DEM on [^{3}H]Taurine Uptake by TR-BBB13 Cells

[^{3}H]Taurine uptake was carried out using TR-BBB13 cells as an *in vitro* BBB model (Terasaki and Hosoya, 2001). The effect of TNF-alpha, LPS, and DEM treatment on [^{3}H]taurine uptake was examined in TR-BBB13 cells and the results are shown in Fig. 1. Treatment with 20 ng/ml TNF-alpha for 24 h resulted in a significant increase in [^{3}H]taurine uptake, while LPS and DEM treatments had no significant effect.. As shown in Fig. 2 (open circles), TNF-alpha pretreatment up to 12 h resulted in an increase in the [^{3}H]taurine uptake by TR-BBB13 cells (165% at 12 h pretreatment). After 24 h treatment, [^{3}H]taurine uptake fell slightly compared with that at 12 h, but was still greater than the uptake without treatment (142% in Fig. 2).

3.2 Effect of Excess Taurine Pretreatment on [^{3}H]Taurine Uptake

The effect of excess taurine on [^{3}H]taurine uptake was examined in TR-BBB13 cells. [^{3}H]Taurine uptake in TR-BBB13 cells exposed to 50 mM taurine for 24 h was reduced by 89.6% in comparison with that under control conditions (Fig. 1). It was significantly reduced in a time-dependent manner up to 24 h (Fig. 2, closed circles).

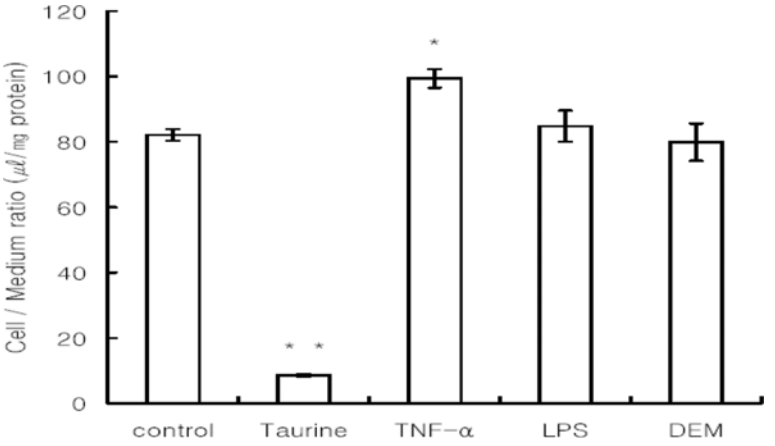

Figure 1. The uptake of [^{3}H]taurine by TR-BBB13 cells under different conditions. Cells were treated with TNF-alpha (20 ng/ml), LPS (10 ng/ml), DEM (100 μM) or taurine (50 mM) for 24 h, respectively. After treatment, taurine uptake was measured after a 5-min incubation with [^{3}H]taurine. Each value represents the mean ± S.E.M. (n=4). *p<0.01, **p<0.0001 significantly different from the control.

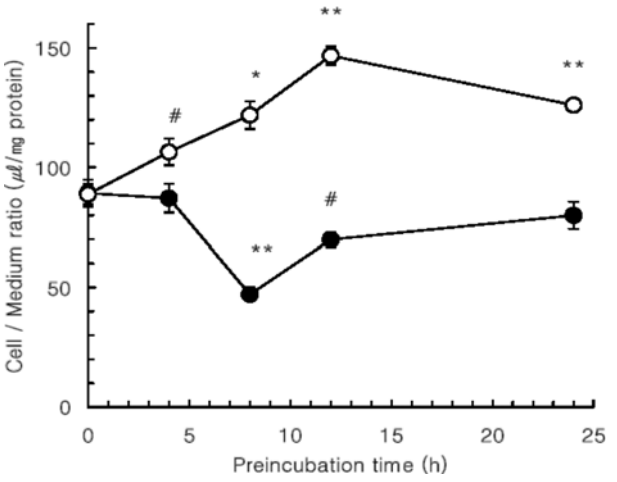

Figure 2. Treatment time-course of TNF-α or unlabelled taurine pretreatment effect on [^{3}H]taurine uptake by TR-BBB13 cells. Twenty ng/ml TNF-alpha (open circle) or 50 mM taurine (closed circle) was preincubated for the time period shown in the figure. Each point represents the mean ± S.E.M. (n=3-4). #p <0.05; *p <0.01, **p <0.001 significantly different from time 0.

3.3 Induction and Suppression of TAUT mRNA Expression in TR-BBB13 Cells Produced by TNF-α, Taurine and Hypertonic Conditions

TAUT mRNA was quantitated by real-time RT-PCR and the results are summarized in Table 3. The TAUT mRNA level in TR-BBB13 cells was increased 3.0-fold following

24 h of TNF-α treatment in comparison with the control. In contrast, after 50-mM taurine treatment for 24 h, the TAUT mRNA level fell by 61.4%.

Table 1. Effects of taurine or TNF-alpha on the mRNA expression of TAUT in TR-BBB13 cells

Treatment	Relative amount [Ratio (TAUT/beta-actin)]
Control	3.74 ± 0.91
Taurine	1.44 ± 0.16*
TNF-alpha	11.4 ± 5.6

Cells were treated with taurine (50 mM) or TNF-alpha (20 ng/ml) for 24 h. The mRNA levels were quantified in each sample in relation to that of beta-actin. Each value represents the mean ± S.E.M. (n=4). *$p<0.05$; significantly different from the control.

4. DISCUSSION

The present study demonstrates that TR-BBB13 cells, an *in vitro* model of the BBB, possess [^{3}H]taurine transport activity. Under certain brain conditions, such as ischemia and brain injury, the taurine level in the brain is increased. *In vivo* brain microdialysis has shown that under ischemic conditions, the taurine level in the brain interstitial fluid is increased 17-fold (from 1.6 μM to 25 μM) (Nakane *et al.,* 1998). Since taurine exerts a neuroprotective effect, this taurine increase is thought to prevent neuronal cell damage or cell death. Taurine uptake by human intestinal Caco-2 cell has been reported to be up-regulated by TNF-alpha (Mochizuki *et al.,* 2002). However, the regulation at the BBB is still unknown. To evaluate the hypothesis that taurine transport at the BBB is regulated by CNS cell damage, the effects of TNF-alpha, LPS, and DEM, cytokine-induced by cell damage, bacterial endotoxin, and compound inducing oxidative stress, respectively, on [^{3}H]taurine uptake by TR-BBB13 cells was investigated. It was found that TNF-alpha induced [^{3}H]taurine uptake by TR-BBB13 cells (Figs. 1 and 2). TNF-alpha is induced in the brain after cerebral ischemia and traumatic brain injury (Shohami *et al.,* 1994, 1999; Meistrell *et al.,* 1997). Therefore, taurine transport at the BBB can be up-regulated by TNF-alpha in response to CNS cell damage. One possible physiological mechanism is that up-regulated BBB taurine transport increases taurine influx from blood to the brain and facilitates an increase in the brain taurine level for neuroprotection. The mechanism of neuroprotective effect is still unclear but may be related to intracellular calcium regulatory action of taurine (Chen *et al.,* 2001).

As shown in Figs. 1 and 2, taurine pretreatment suppresses [^{3}H]taurine uptake by TR-BBB13 cells. This suppression appears to act as a feed-back mechanism for the regulation of taurine transport activity at the BBB. Taurine in the brain comes from the circulating blood as well as biosynthesis in the brain (Huxtable and Lippincott, 1982). Therefore, one possible mechanism is that the plasma taurine concentration (10-100 μM) (Huxtable, 1992) regulates taurine transport activity at the BBB. Another possible mechanism is that increased taurine levels in the brain suppress BBB taurine transport activity, which is induced by other factors, such as TNF-alpha. After rise of brain taurine under ischemic conditions, its level returns to normal (Nakane *et al.,* 1998). This decrease is likely due to the limited supply of taurine from blood and uptake by neurons and astrocytes.

Transcriptional regulation is involved in taurine transport activity in TR-BBB13 cells, since induction and suppression of taurine uptake activity was associated with an increase and decrease in TAUT mRNA level, respectively, as shown in Table 1. The TNF-alpha signal activates NF-κB transcriptional activity by nuclear translocation, and the NF-κB binding site (-458 GGGGCTCCC -466; consensus sequence GGGRNTYYC) is found in the TAUT promoter region (GenBank accession number AF151716) (Han *et al.,* 2000). Hence, the TNF-α-NF-κB pathway could be involved in TAUT induction. Beside transcriptional regulation of TAUT, post-translational regulation of TAUT transport activity has been reported. TAUT transport activity is down-regulated by protein kinase C (PKC) activation in *Xenopus* oocytes (Loo *et al.,* 1996), and serine-322 in TAUT is a critical site of PKC regulation (Han *et al.,* 1999). Therefore, the involvement of post-translational regulation in TAUT at the BBB is also considerable, although further studies are necessary to clarify this.

It has been reported that taurine is released from hippocampal slices under conditions of cell damage, such as hypoxia, ischemia, oxidative stress, and hypoglycemia (Saransaari and Oja, 2000). This result suggests that taurine release from neuronal cells also plays a role in increasing brain taurine levels following CNS cell damage. In ischemia, an increase in the taurine level in the brain interstitial fluid was observed within 20 min (Nakane *et al.,* 1998), although the taurine uptake activity in TR-BBB13 cells was increased by 1.2-fold after 5 h of TNF-alpha treatment (Fig. 2). In contrast, taurine release from hippocampal slices was observed within 50 min (Saransaari and Oja, 1997). Therefore, one possible explanation is that taurine release from neural cells contributes to the acute taurine increase, and the taurine transport system at the BBB is involved in regulation of brain taurine levels after this acute increase.

The taurine transport system is present in both the luminal and abluminal membranes of the BCEC, and the luminal uptake rate is about 2-fold greater than that of abluminal uptake (Tamai *et al.,* 1995; Tsuji and Tamai, 1996). Blood-to-brain transport would be involved in supplying taurine from the circulating blood and increasing the brain taurine level, while brain-to-blood transport possibly contributes to taurine elimination and reduction in the brain taurine level. The regulation of taurine transport by TAUT expression can influence either or both directions of taurine transport at the BBB, and it is important to clarify which side of taurine uptake is regulated by TNF-alpha and taurine. It has been considered that the apical surface of the cultured cells corresponds to the luminal side of brain capillary endothelium, but TR-BBB cells exhibited system A transport activity, which exists on the abluminal side, as shown by a cellular uptake study (Takanaga *et al.,* 2002). This observation suggests that the apical surface of TR-BBB cells also possesses the abluminal properties of the BBB. Therefore, the transport regulation in TR-BBB cells shown in the present study is likely to reflect not only that on luminal side but also that on abluminal side for at least to some part. Further studies are needed to obtain more information about this directional regulation of taurine transport.

In conclusion, mRNA expression of TAUT and taurine transport activity were induced following TNF-alpha treatment in TR-BBB13 cells while they were reduced following treatment with excess taurine. These findings provide us with a better understanding of the novel BBB function involved in controlling brain taurine levels and the mechanism for protecting neuronal cells against stress and other damage.

5. ACKNOWLEDGMENT

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NEUROPROTECTION BY TAURINE AND TAURINE ANALOGUES

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1. INTRODUCTION

The anti-inflammatory action of taurine and its analogues is attributable to three major pathways: (a) its reaction with hypochlorous acid to form the less toxic anti-inflammatory mediator; taurine chloramine (reviewed in Della Corte *et al.,* 2002), (b) its modulatory effect on NMDA-evoked glutamate release (Dachour and De Witte, 2003), and (c) its role in calcium homeostasis possibly mediated by enhancing calcium uptake into mitochondria (Palmi *et al.,* 1999). The concentration of taurine in phagocytic cells, such as microglia and macrophages, is in the mM range, (Banks *et al.,* 1991) which decreases when such cells are stimulated, due to phosphokinase C (PKC)-induced down-regulation of the taurine transporter TAUT (Tappaz, 2004), thereby prolonging the effect of hypochlorous acid. Microglia will also remove potentially toxic glutamate from the synaptic space (Mark *et al.,* 2001) and release various mediators to combat the inflammatory stimulus.

In certain neurological lesions, e.g Parkinson's and Alzheimer's diseases, there are chronic inflammatory stimuli exacerbating neurotoxicity that may be beneficial to diminish the action of hypochlorous acid and by increasing cellular taurine content. However, high doses of taurine, >2 g, are needed to be administered in order to override the controlled taurine uptake by its transporter, TAUT. Furthermore increasing cellular taurine homeostasis may initiate down-regulation of TAUT, thereby diminishing any beneficial effect of the initial supplementation after a short-time period. Therefore the development of taurine analogues which are able to penetrate the cell membrane

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Taurine 6
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independent of TAUT may be of therapeutic advantage in such diseases. Fig. 1 shows the chemical structures of the taurine analogue tauropyrone.

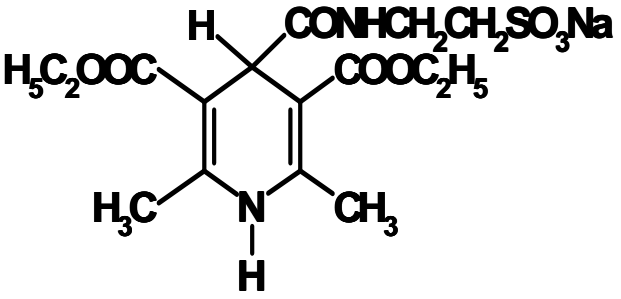

Figure 1. Chemical structure of the taurine analogue tauropyrone.

In the present study the ability of taurine to enhance antioxidant protection over a two-week time period has been studied, while the ability of taurine and a taurine analogue, tauropyrone, to diminish an inflammatory response has been studied both *in vitro* in an LPS-IFNγ stimulated microglial cells line and *in vivo* in the 6-OH-dopamine (6-OHDA) model of Parkinson's disease.

2. MATERIAL AND METHODS

2.1. Animal Studies

Male Sprague-Dawley rats (n=12) were administered taurine (12.5 g/l) in their drinking bottles for 7 and 14 days. At the end of these two time points, the rats were killed by cervical dislocation and livers removed. These were homogenized in 0.25 M sucrose (10% w/v), centrifuged at 3,000 rpm and the activities of catalase and superoxide dismutase as well as glutathione content assayed in the supernatant.

Male Sprague-Dawley rats, starting weights of 225±25 g, were housed in groups of three with free access to food and water, under controlled temperature and a 12:12 h light:dark cycle, (lights on 07:00). Taurine (12.5 g/l) or the taurine analogue tauropyrone (6.25 g/l) was dissolved in water and placed in the water bottle to which the rats had free access. Sucrose (2%) was also added to the taurine analogue tauropyrone solution to encourage its intake by the rats. After 4 days, rats were anaesthetized with isofluorane and 6-OHDA (12 μg dissolved in 4 μl 0.1% ascorbic acid/saline solution) injected onto the medial forebrain bundle (stereotactic co-ordinates: 2.2 mm anterior, +1.5 mm lateral from bregma and –7.9 mm ventral to dura from ear bars 5 mm below incisor bar) (Paxinos and Watson, 1986; Datla *et al.,* 2001). The animals received further 3 days drug treatment after which they received normal drinking water. Seven days after the 6-OHDA lesions, the rats were killed by cervical dislocation and the brains dissected immediately. A coronal section was made at the level of hypothalamus and the forebrain and hindbrain parts were separated. The hindbrain was fixed for 7 days in 4%

paraformaldehyde, cryoprotected with 30% sucrose solution for 2-3 days and used for tyrosine hydroxylase (TH) immunostaining as previously described (Datla *et al.,* 2001). TH was immunostained by incubating the 20-mm fixed coronal free-floating sections with polyclonal rabbit anti-TH (1:3000, Chemicon,UK) followed by biotinylated anti-rabbit IgG and avidin/biotin complex (Vector Lab, UK). The TH immunocomplex was then visualized by diaminobenzidine and H_2O_2. Images of the TH^+ cells were captured by a Xillix CCD digital camera and counted manually at level B in the substantia nigra pars compacta according to Carman *et al.* (1991). From the forebrain, lesioned and control striata were dissected out and assayed for dopamine (DA) and its metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) by HPLC with electrochemical detection (Datla *et al.,* 2001).

2.2. Cell Culture Studies

An immortilised glial cell line BV2 was cultured in RPMI + 10% fetal calf serum, 1% glutamine, 1% penicillamine/streptomycin, which was supplemented with taurine, tauropyrone or homotaurine, 100 μM. An inflammatory stimulus, lipopolysaccharide (LPS) 1 mg/ml +/- interferon gamma (IFNγ) 1 U/ml, was administered for a 44-h period, when the LPS-NO evoked release was assayed in the culture medium. A 100-μl sample was incubated with an equal volume of Griess reagent (1% sulphanilamide; 0.1% N-1-naphthyethylenediamine dihydrochloride; 2.5% H_3PO_4) at room temperature for 10 minutes. The absorbance at 550 nm was read by a microplate reader. Nitrite standards in the appropriate reference range (0-50 μM) were prepared from sodium nitrite.

2.3. Cytoprotective Enzymes and Antioxidants

2.3.1. Superoxide Dismutase

Superoxide dismutase (SOD) was analysed by the method of Beachamp and Fridovich (1971). Superoxide is generated *in vitro* via the xanthine/xanthine oxidase reaction which reduces the color reagent nitroblue tetrazolium (NTBB). The SOD in the sample competes with the NTBB for the superoxide. The activity of SOD is inversely proportional to the intensity of colour produced. One unit of SOD corresponds to an inhibition of 50% in the reduction of NTBB by superoxide.

2.3.2. Catalase

Catalase was assayed by the method of Peters and Batt (1976) in which an *in vitro* source of hydrogen peroxide is scavenged by catalase for a known time period. Thereafter, a color reagent, titanium peroxysulphate, is added which reacts with any remaining peroxide. Spectrophotometric analysis is made at 405 nm, and the intensity of the color is inversely proportional to the catalase activity in the sample.

2.3.3. Glutathione

Reduced glutathione (GSH) was determined by a modification of the fluorometric method of Hissin and Hilk (1976). GSH react with o-phthaldehyde at pH 8 after its complexation with N-ethylmaleimide to prevent interference with the assay from

oxidized glutathione (GSSH). Fluorescent intensity is measured at 420 nm with excitation at 350 nm.

2.4. Statistical Analyses

The results are presented as mean values ± standard deviation. Statistical analysis was made by two-way ANOVA with significance verified by Fisher 't' test.

3. RESULTS

Significant increases in SOD activity and glutathione content were assayed in the livers of the taurine-supplemented rats at 7 days when compared to the controls rats, although no changes were evident at 14 days (Fig. 2). The hepatic catalase activity significantly increased after 7 and 14 days of taurine supplementation.

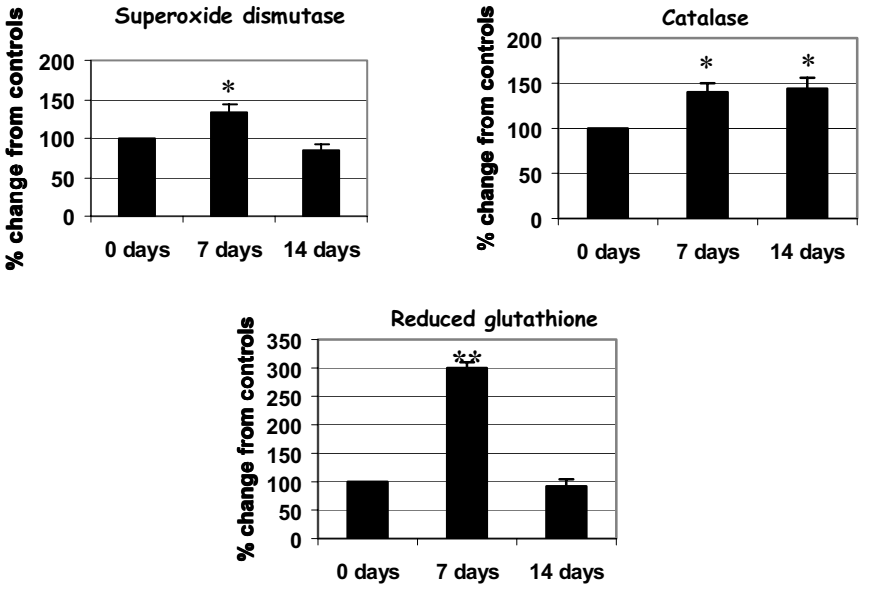

Figure 2. Activities of superoxide dismutase and catalase and the glutathione content in the liver after 7 days or 14 days of oral taurine supplementation.

Lesioning one half of the brain with 6-OHDA significantly reduced the number of tyrosine hydroxylase cells TH^+ when compared to the control side (Fig. 3, vehicle). However, administration of either taurine or tauropyrone to the 6-OHDA rats significantly reduced the loss of TH^+ cells in the lesioned side (Fig. 3a). The levels of dopamine were decreased significantly by lesioning, while the loss of this monoamine was considerably less after administration of taurine, $P<0.05$ (Fig. 3b). The results for

tauropyrone, when the mean loss of dopamine was reduced, were not significant since the standard deviation was large.

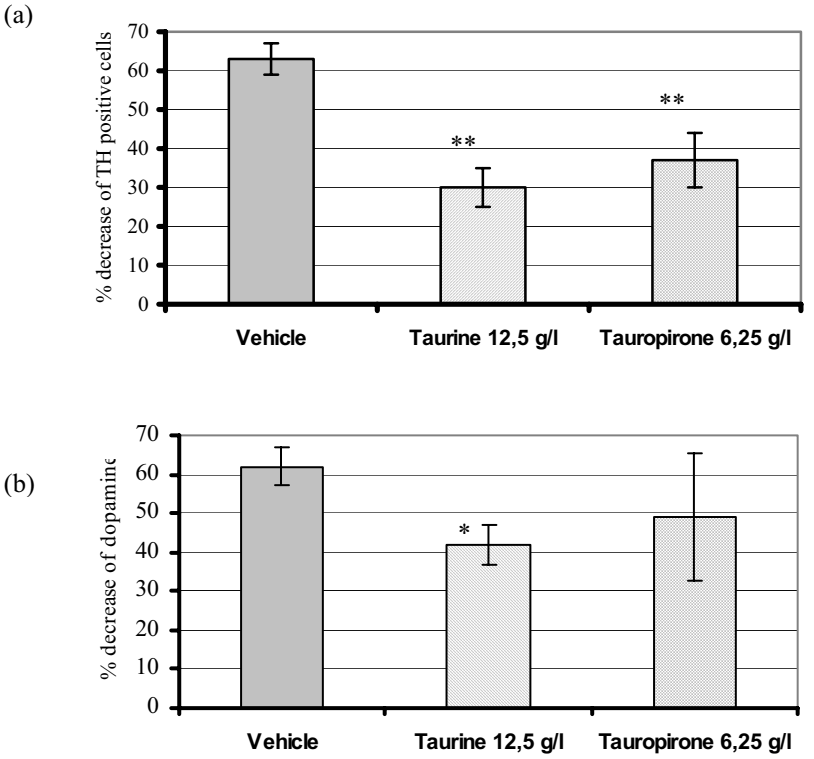

Figure 3. Beneficial effects of taurine or tauropyrone supplementation on (a) loss of TH+ cells and (b) dopamine synthesis in the 6-OHDA animal model.

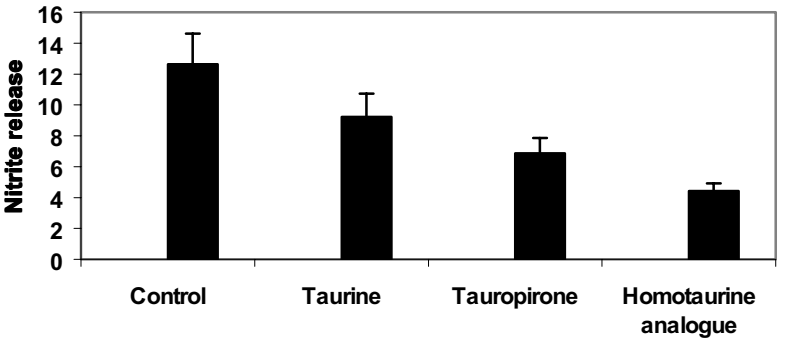

Figure 4. Effect of supplementation of taurine, tauropyrone and homotaurine on the LPS-evoked NO release in BV2 glial cells.

Incubation of BV2 cells with taurine, tauropyrone or homotaurine prior to the LPS-IFNγ inflammatory stimulus significantly reduced the LPS-evoked NO release when compared to controls (Fig. 4).

4. DISCUSSION

It was noteworthy that the increase in mean hepatic superoxide dismutase activity and the glutathione content, which was evident after 7 days of taurine supplementation 2.85 g/kg/day, was lost after 14 days, indicating an alteration in taurine homeostasis at this time point. The total taurine content, although increasing approximately-2 fold after taurine supplementation, maintains its levels for the subsequent 30 days of supplementation indicating a saturation level for its uptake by the liver (Ledeque, 2003). Further subcellular investigation may help to elucidate the changes in the taurine pools that occur between the different organelles during the period of taurine supplementation. *In vivo* administration of taurine; 0.5 g/kg/day, to rats in which inflammatory bowel disease (by trinitrobenzene sulfonic acid) had been induced, also showed an increase in defenses against oxidative insult (Son *et al.,* 1998).

The beneficial effects of both taurine and tauropyrone supplementation in preventing progression of the neurodegenerative/inflammatory process in the 6-OHDA model would indicate that both of these compounds had crossed the blood brain barrier. The 6-OHDA model of Parkinson's disease shows similar biochemical features to Parkinson's disease where the loss of TH^+ cells is progressive and associated with an inflammatory response (Perese *et al.,* 1989). Indeed, auto-oxidation of dopamine via 6-OHDA may be of importance in the development of Parkinson's disease, the oxidized metabolite causing toxicity and neuronal death (Crichton and Ward, 2005). It is reputed that 6-OHDA needs to be transported into the dopaminergic neurons via its transporter for neurotoxicity to be manifested. Inhibition of this uptake e.g. by estrogens (Dluzen, 2000) can be neuroprotective. The beneficial action of tauropyrone at a 50% lower dose that taurine in diminishing neurotoxicity might indicate that it was able to traverse the cellular membrane independently of TAUT due to its lipophilic structure. Further kinetic studies will evaluate how tauropyrone traverses the membrane. The decrease in neurotoxicity may be caused by an enhancement of brain cytoprotection and glutathione content (in an analogous way to that in the liver) as well as by taurine-induced changes in calcium fluxes.

Taurine, tauropyrone and homotaurine reduced the LPS-stimulated release of NO, homotaurine being the most efficient. LPS will induce the transcription and translation of iNOS with the formation of NO to destroy the invading pathogens. Such formation is under the control of the transcription factor NFkappaB, a key orchestrator in the inflammatory process. Hence, a reduction in its activation would reduce iNOS formation. Tau-Cl depresses NFkappaB migration into the nucleus of various cell lines: e.g in TNF-α activated primary and immortalized rat alveolar macrophages (NR8383 cells) by means of decreased phosphorylation of alpha serine 32 in IkappaBα and a lower activity of IkappaB kinase (Barua *et al.,* 2001) and in IL-1 activated leucocytes by oxidation of IkappaBα at Met45 (Schuller-Levis and Park, 2003). In previous studies, taurine chloramine, but not taurine, inhibited pro-inflammatory mediators such as nitric oxide and prostaglandin E2 in activated rodent macrophages (Chorazy *et al.,* 2002) and activated C6 glioma cells. (Liu *et al.,* 1999) as well as suppressing superoxide anion, IL-6

and IL-8 in activated human polymorphonuclear leukocytes and lymphocyte proliferation (Park *et al.,* 2002). However, it is unclear why the taurine supplementation experiments were ineffective in decreasing inflammation since it would be expected that taurine chloramine would be synthesised by non-enzymatic processes (Olszowski *et al.,* 2002). Inflammation *in vitro*, induced by PMA or LPS in murine macrophages RAW 264.7, will alter taurine homeostasis, in part by decreasing taurine transporter activity (Kim *et al.,* 2003) and by inducing changes in the activities of taurine biosynthetic enzymes, cysteine dioxygenase and cysteinesulphinate decarboxylase. Further studies on changes in microglial taurine homeostasis after stimulation +/- taurine analogues are needed to clarify the role played by these analogues.

In these present studies taurine analogues have been shown to be superior to taurine in retarding the inflammatory process both *in vivo* in an animal model of Parkinson's disease and *in vitro* in a microglial cell line. Further development of such compounds may represent a new therapeutic approach for the retardation of neurological diseases where inflammation is known to be an important factor in the progression of neurotoxicity.

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TAURINE TRANSPORTER REGULATION IN HIPPOCAMPAL NEURONS

James E. Olson and Eduardo Martinho, Jr.*

1. INTRODUCTION

Taurine, one of the most prevalent free amino acids in the brain and other tissues (Awapara *et al*., 1950), has been widely studied as an osmolyte used for regulation of brain cell volume (Pasantes-Morales *et al*., 2000b). Taurine is produced metabolically from cystine in brain cells (Peck and Awapara, 1967); however, this is a slow process, requiring approximately 48 h to replace the total cellular content of cerebral astrocytes (Beetsch and Olson, 1998). In contrast, active taurine accumulation via a plasma membrane transporter in these cells is approximately 30 times faster than the metabolic pathway (Beetsch and Olson, 1996). Furthermore, passive efflux of taurine via plasma membrane channels can reduce cellular taurine contents by as much as 5% per min in osmotically swollen cells and can deplete cells of their taurine contents in only 20-30 min (Kimelberg *et al*., 1990; Pasantes-Morales *et al*., 1990). Thus, minute to minute regulation of cellular taurine content is achieved by the balance of these passive and active membrane transport systems.

Rates of taurine influx and efflux are equal in steady state conditions resulting in constant intracellular taurine and water contents (Fig. 1A). Imposed changes in cell volume can alter the rate of taurine movements across plasma membranes. For example, osmotically swollen glial cells activate the efflux pathway and thus, rapidly lose taurine into the extracellular space (Kimelberg *et al*., 1990; Pasantes-Morales *et al*., 1990). This channel activation is mediated by intracellular kinase pathways including ERK1/2 and PI3 (Crepel *et al*., 1998; Pasantes-Morales *et al*., 2000a). Extracellular purinergic signaling also has been implicated for this channel activation in glial cells and neurons (Darby *et al*., 2003; Li and Olson, 2004). The resulting efflux of taurine would lead to a reduction of cell volume by the loss of osmotically obliged water.

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Taurine 6
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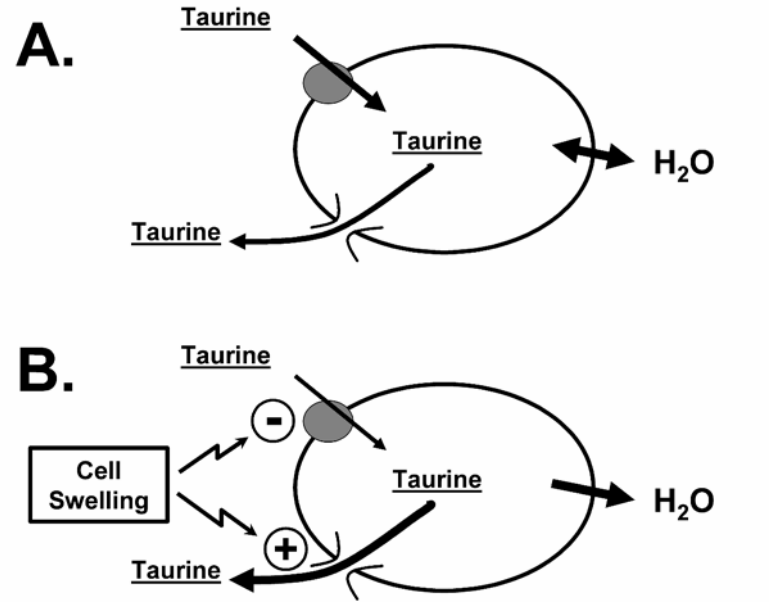

Figure 1. A. Steady state cellular taurine content is achieved when rates of influx via the taurine transporter (shaded circle) and efflux via membrane channels are equal. In this condition, no osmotic water flow will be observed. **B.** We hypothesize cell swelling causes downregulation of the taurine influx pathway via the plasma membrane transporter as well as increased taurine efflux via membrane channels. This results in a net loss of taurine and osmotically obliged water.

In contrast, osmotically shrunken cells increase taurine accumulation via the plasma membrane taurine transporter (Beetsch and Olson, 1993). Cells may increase transport activity by elevated gene expression (Bitoun and Tappaz, 2000) or by insertion of preformed transporter protein into the plasma membrane (Chesney *et al*., 1989).

Changes in taurine transport measured in swollen brain cells *in vitro* are consistent with *in situ* observations. Edematous brains show an increased extracellular taurine concentration and diminished taurine contents (Solis *et al*., 1988; Wade *et al*., 1988; Verbalis and Gullans, 1991; Bedford and Leader, 1993). Increased efflux will lead to neuronal taurine loss *in vivo*; however, the resulting increase in extracellular taurine also will increase taurine accumulation via the membrane transporter. A resulting futile cycle of taurine efflux and accumulation would diminish the net change in taurine content and resulting cell volume regulation. Therefore, we hypothesize that, in addition to activation of efflux pathways, swollen neurons will downregulate the rate of taurine accumulation (Fig. 1B). We chose to study this mechanism in cultured hippocampal neurons because these cells accumulate taurine *in vivo* (Pow *et al*., 2002) and because taurine has been shown to be critical for osmotic regulation in hippocampal slices (Kreisman and Olson, 2003). In these preliminary studies we sought to characterize the kinetic and pharmacological properties of neuronal taurine transport and examine the effect of hypoosmotic swelling on these parameters. Regulation of taurine transport has been examined in a number of cell types including cortical neurons, astroglial cells, kidney cells, and

Table 1. Intracellular signaling pathways involved in taurine transporter regulation

Intracellular signaling pathway	Cell type	Response to pathway activation	Reference
PKC	Renal epithelial cells	Downregulation	(Jones *et al*., 1991)
	Astrocytes	Downregulation	(Tchoumkeu-Nzouessa and Rebel, 1996)
	Cortical neurons	No effect	(Tchoumkeu-Nzouessa and Rebel, 1996)
	Kidney cell transporter expressed in *Xenopus*	Downregulation	(Han *et al*., 1999)
	Aortic endothelial cells	Downregulation	(Qian *et al*., 2000)
	Macrophages	Downregulation	(Kim *et al*., 2003)
	3T3 fibroblasts	Downregulation	(Voss *et al*., 2004)
PP1-3	Ehrlich cells	Upregulation	(Mollerup and Lambert, 1998)
cAMP	Thyroid cells	Upregulation	(Jhiang *et al*., 1993)
	Retinal pigment epithelial cells	Upregulation	(Ganapathy *et al*., 1995)
	3T3 fibroblasts	Downregulation	(Voss *et al*., 2004)
NO	Retinal pigment epithelial cells	Upregulation	(Bridges *et al*., 2001)
TNFα	Astrocytes	Upregulation	(Chang *et al*., 2001)
	Brain endothelial cells	Upregulation	(Kang *et al*., 2002)
	3T3 fibroblasts	Upregulation	(Voss *et al*., 2004)
LPS	Macrophages	Downregulation	(Romio *et al*., 2001; Kim *et al*., 2003)

Abbreviations used: PKC, protein kinase-C; PP1-3, protein phosphatases 1-3; cAMP, 3',5'-cyclic adenosine monophosphate; NO, nitric oxide; TNFα, tumor necrosis factor alpha; LPS, bacterial lipopolysaccharide.

macrophages. These studies have revealed a variety of potential intracellular signaling pathways which may modify the rate of taurine accumulation (Table 1). Thus, we sought to explore which of these possible intracellular signaling pathways might regulate changes in the activity of the taurine transporter in hippocampal neurons.

2. METHODS

Primary cultures of hippocampal neurons were prepared from rat fetuses as previously described (Li and Olson, 2004). Cells were grown for 10-14 days before experimentation. Cell counts of cultures doubled stained for antibodies directed against neuron specific enolase (Sigma-Aldrich Chemical Company, St. Louis, Missouri) and glial fibrillary acidic protein (Sternberger Monoclonals, Baltimore, Maryland) revealed that approximately 78% of the cells were neurons. Cultures immunostained with antibodies

Table 2. Calculated kinetic parameters for taurine accumulation into hippocampal neuron cultures incubated in isoosmotic and hypoosmotic PBS

	J_{max} nmol/(mg protein min)	K_m μM	K_{diff} ml/(mg protein min)
Isoosmotic	1.58 ± 0.23	57 ± 22	0.00064 ± 0.00015
Hypoosmotic	0.82 ± 0.22*	38 ± 29	0.00113 ± 0.00016*

Values are the mean ± SE of the parameters calculated using a non-linear least squares method. * indicates parameters which are significantly different from the value measured in isoosmotic conditions.

directed against the taurine transporter (TauT, Chemicon International, Temecula, California) demonstrated strong labeling of neurons and light staining of glial cells. Therefore we anticipate the majority of carrier-medicated taurine accumulation in these cultures is due to transporter activity at the neuronal plasma membrane.

Taurine uptake was measured as the accumulation of [^{3}H]taurine (Perkin Elmer, Boston, Massachusetts) by cell cultures incubated with concentrations of unlabeled taurine ranging between 1 μM and 2000 μM. Unless noted otherwise, studies were performed at 37^{o}C in isoosmotic phosphate buffered saline (PBS) consisting of 137 mM NaCl, 2.7 mM KCl, 1.0 mM $CaCl_2$, 0.5 mM $MgCl_2$, 2.7 mM Na_2HPO_4, 0.5 mM KH_2PO_4, and 5.5 mM glucose (pH 7.3). Small volumes of 3 M NaCl were added to adjust the osmolality to 290 mOsm. The quantity of accumulated taurine by each culture dish was normalized to the protein content of the dish determined by the method of Lowry et al. (1951). When drugs were present, they were added to isoosmotic PBS incubation solution 30 min prior to the introduction of radioactive taurine tracer. Hypoosmotic PBS (200 mOsm) was prepared by reducing the concentration of NaCl. In some experiments, the concentration of sodium was reduced by equimolar substitution with choline. Kinetic data were fit to theoretical relationships using a non-linear iterative method.

3. RESULTS

Accumulation of 100 μM taurine by the neuronal cultures at 37^{o}C was linear for at least 30 min with a rate of 1.46 ± 0.13 nmol/mg protein min (mean ± SE). The rate of accumulation was diminished to 1.5% of this value for cells incubated at 0^{o}C. The kinetic curve relating extracellular taurine concentration to taurine uptake was consistent with accumulation mediated by parallel saturable and non-saturable components. The saturable component had a calculated J_{max} of 2.1 ± 0.23 nmol/mg protein min and a K_m of 30 ± 8.8 μM while the non-saturable component had a K_{diff} of 0.0013 ± 0.0002 ml/min.

At an extracellular concentration of 1 μM, taurine will be accumulated predominantly by the saturable component. At this extracellular concentration, taurine accumulation was strongly inhibited by 1 mM of 2-guanidinoethanesulphonate, β-alanine, or hypotaurine, but only slightly reduced by 1 mM glutamine, glutamate or betaine. Accumulation of 1 μM taurine by the neuronal cultures also showed a strong dependence on extracellular sodium. The relationship between sodium concentration and the rate of taurine

accumulation was sigmoidal with a calculated Hill coefficient (± SE) of 2.1 ± 0.1 and a K_m for sodium of 81.4 ± 1.6 mM.

Because of the high sodium dependence of taurine accumulation, the effect of osmolality on taurine transport was determined in isoosmotic and hypoosmotic solutions containing the same concentration of NaCl (100 mM). Osmolality of each solution was adjusted by the addition of sucrose. As shown in Table 2, taurine accumulation in hypoosmotic conditions showed a decrease in J_{max}, an increase in K_{diff}, and no change in K_m compared with cells in isoosmotic conditions.

A variety of pharmacological agents which modify intracellular signaling pathways were examined for their effect on the rate of transport of 1 μM taurine. Phosphatase inhibition by 100 μM orthovanadate slightly decreased the rate of taurine transport in isoosmotic conditions, but did not alter the response to hypoosmotic exposure. In contrast, 1 mM genistein, an inhibitor of protein kinase activity, had no effect on taurine transport in isoosmotic conditions; however, it completely blocked the hypoosmotic-induced decrease in taurine transport seen in control conditions without added drug.

The role of PKC for the regulation of neuronal taurine transport was studied using a number of drugs which increase (30 min exposure to 1 μM phorbol myristate acetate, PMA) or decrease (24 h exposure to 1 μM PMA or 30 min exposure to 3 μM phorbol-12,13-dibutyrate, 2.5 μM chelerythrine, 10 μM Gö6983 or 10 μM Gö6996) PKC activity. None of these treatments altered the rate of taurine accumulation in either isoosmotic or hypoosmotic conditions compared with the rate measured in control cells without added drug. Similarly, agonists of the adrenergic pathway including 50 μM noradrenaline or 100 μM of either dibutyryl-cAMP, phenylephrine, or isoproterenol had no effect on the rate of accumulation of 1 μM taurine in either isoosmotic or hypoosmotic conditions.

4. DISCUSSION

Our data demonstrate cultured hippocampal neurons express the taurine transporter. The characteristics of taurine accumulation in this cell system are similar to that described for astrocytes, Ehrlich ascites tumor cells, cultured granule neurons, and lens epithelial cells (Schousboe *et al*., 1976; Holopainen *et al*., 1987; Beetsch and Olson, 1993; Mollerup and Lambert, 1998; Cammarata *et al*., 2002). Kinetics of taurine accumulation in these cultured neurons is consistent with a saturable transporter obeying Michaelis-Menten kinetics in parallel with a non-saturable transport mechanism. The saturable component, representing taurine transporter activity, has a calculated binding coefficient for taurine of around 30 μM. This value is within the 7 μM to 90 μM range of taurine transporter affinities described for other cell systems (Schousboe *et al*., 1976; Holopainen *et al*., 1987; Sanchez-Olea *et al*., 1991; Beetsch and Olson, 1993; Mollerup and Lambert, 1998; Bridges *et al*., 2001; Romio *et al*., 2001; Cammarata *et al*., 2002; Takahashi *et al*., 2003; Voss *et al*., 2004). Furthermore, as we previously described for cultured astrocytes (Beetsch and Olson, 1993), taurine uptake in the cultured neurons was inhibited by low temperature suggesting strong energy dependence for taurine accumulation. Analysis of the kinetic components indicates that at 1 μM extracellular taurine, 98% of the accumulation is mediated via the saturable transporter. With this concentration of extracellular taurine, uptake is strongly inhibited by structural analogs of taurine and is

dependent on extracellular sodium with a calculated Hill coefficient close to 2. These characteristics also are similar to those reported for the taurine transporter in other tissues (Schousboe *et al*., 1976; Holopainen *et al*., 1987; Uchida *et al*., 1991; Barakat *et al*., 2002; Takahashi *et al*., 2003; Voss *et al*., 2004).

The identity of the non-saturable component of taurine accumulation is not clear from these studies; however, for other cell types, this component has been suggested to represent taurine movement through a diffusion pathway (Sanchez-Olea *et al*., 1991). We have observed anion and taurine conductances in these cells (Li and Olson, 2004) similar to osmolyte conducting pathways described in glial cells and other cell types (Jackson and Strange, 1993; Roy and Banderali, 1994; Olson and Li, 1997). Membrane taurine conductance of cultured hippocampal neurons is increased by hypoosmotic swelling and extracellular ATP (Li and Olson, 2004). In the current studies, we observed an increase in the non-saturable component of taurine accumulation in osmotically swollen neuron cultures. This parallels previous electrophysiological studies on taurine conductance and indicates at least a portion of the non-saturable accumulation pathway is mediated by taurine-conducting membrane channels.

Osmotically swollen neurons showed a decrease in J_{max} for the saturable component of taurine accumulation coincident with an increase in the K_{diff} for the non-saturable component. If K_{diff} reflects taurine movements through membrane channels, these changes in taurine transport would result in a net loss of taurine from neurons. Since the maximal rate of the astroglial taurine transporter is not elevated by osmotic swelling (Sanchez-Olea *et al*., 1991), these changes in neuronal taurine transport occurring *in vivo* could result in net transfer of taurine and osmotically obliged water from neurons into glial cells. Evidence for movement of neuronal taurine into glial cells has been observed in the cerebella of rats during hypoosmotic hyponatremia (Nagelhus *et al*., 1993).

Similar to data from cultured cortical neurons (Tchoumkeu-Nzouessa and Rebel, 1996), PKC does not modify taurine transporter activity in the hippocampal cultures. We also found no evidence for regulation of the transporter by PKA. However, significant effects were obtained using genistein and orthovanadate, broad pharmacological inhibitors of protein kinases and phosphatases, respectively. From these results, we conclude downregulation of the neuronal taurine transporter in hypoosmotic conditions is mediated by protein phosphorylation. Several potential sites for phosphorylation have been observed in the cloned sequence of the taurine transporter described for canine kidney cells and one of these has been associated with downregulation of the transporter by PKC (Han *et al*., 1999). Identifying the phosphorylation site responsible for osmotic regulation of the neuronal taurine transporter and the intracellular signaling pathways involved in this alteration in activity must await further experimentation.

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TAURINE AND BRAIN EXCITABILITY

Abdeslem El Idrissi*

1. INTRODUCTION

The goal of this study is to characterize neuronal plasticity of the GABAergic system induced by chronic supplementation of taurine. Our preliminary data indicate that during early periods of developmental maturation, the brain is very sensitive to environmental factors that affect neuronal excitability. These early "critical periods" of brain development correspond to a time of maximal synapse elaboration, complex pathway formation, and the time of optimal seizure sensitivity. Possible developmental processes related to seizure propensity in the neonatal brain may be due to the relatively low levels of inhibition, peak NMDA receptor concentration, and sub-optimal regulation of extracellular ion levels due to immature activities of relevant pumps.

We have shown previously that chronic supplementation of taurine in drinking water induces a state of brain excitability characterized by increased susceptibility to kainic acid (KA) -induced seizures. This reduction in seizure threshold was demonstrated by a decreased latency for the onset of clonic seizures, an increased incidence and duration of tonic-clonic seizures, increased neuronal death in the CA3 region of the hippocampus, and a higher post-seizure mortality rate of the animals. KA causes severe convulsions in mice and has been used as a rodent model for human temporal lobe epilepsy. Using this paradigm of taurine treatment to induced hyper-excitability, we examined changes that occur in the GABAergic system as a possible compensatory mechanism for the increased excitability. We found that taurine-fed mice have elevated brain levels of glutamate and GABA. This increase in neurotransmitter levels was accompanied by an increase in the expression of GABA synthesizing enzyme, glutamate decarboxylase (GAD). Furthermore, taurine-fed mice have reduced expression of $GABA_A$ receptors in the hippocampus.

We propose that chronic supplementation of taurine in drinking water induces a down-regulation of $GABA_A$ receptors expression due to the sustained interaction of taurine with $GABA_A$ receptors which decreases the efficacy of the inhibitory synapses at postsynaptic membrane. As a compensatory mechanism to this increased excitability,

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there is increased GAD expression on the pre-synaptic side. This is demonstrated by the increased expression of both isoforms of this enzyme in inhibitory interneurons. Concomitant with this increased expression of GAD there was an increase in the levels of the neurotransmitter GABA in the brain. It should be noted, however, that the excitability induced by taurine is compatible with normal brain function and sub-threshold to induce spontaneous seizures. In this model of seizure KA injections are used to induce seizures and the threshold for seizure induction is used to determine the excitability levels.

2. MATERIAL AND METHODS

2.1. Cerebellar Granule Cell Culture

Cerebellar granule cells were prepared from 7-day-old mice as previously described (Trenkner and Sidman, 1977; Trenkner, 1991; El Idrissi and Trenkner, 1999). The cultures were maintained in a humidified CO_2/air (5%/95%) atmosphere at 37°C and monitored daily by phase contrast microscopy.

2.2. $^{36}Cl^-$ Influx Measurements

Cerebellar granule cells were cultured for 5 days *in vitro*. Growth medium was poured off and cultures were washed three times with Earl's balanced salt solution (EBSS). After equilibration in 1 ml of EBSS for 20 min at 37°C, $^{36}Cl^-$ influx measurements were initiated by replacing this solution with an identical one containing 5 mCi/l $^{36}Cl^-$, GABA and taurine. Cells were incubated in the presence of ligands for 10 s. After that, free radioligand was removed by three rapid (5 s) rinses with EBSS. The radioactivity was extracted by 2 x 0.5 ml distilled water followed by 2 x 0.5 ml methanol and counted by standard liquid scintillation spectrometry.

2.3. Drug Administration

Taurine was provided in drinking water (0.05% w/v) for one month before perfusing the mice and collecting the brain for analysis. All mice used in these experiments were two months old and supplementation of taurine in drinking water started at four weeks of age.

2.4. Western Blotting

Brains were dissected within three minutes of the sacrifice and frozen on dry ice. Total soluble and membrane bound proteins were extracted from each region (Benke *et al.,* 1999). For γ-aminobutyric acid (GABA) receptor β subunit (GABARB) detection 40 μg of protein from each region was loaded onto a 12.5% polyacrylamide-SDS gel and transferred onto PVDF membrane (Millipore, Bedford, MA) after electrophoresis. Membranes were sequentially probed with two monoclonal antibodies: MAB341 (1:1000 dilution, Chemicon, Temecula, CA) that recognizes all subtypes of GABARB and an antibody (clone AC-15, Sigma, St. Louis, MO) that recognizes β-actin (1:200,000 dilution) as a control for protein loading. For GAD detection 10 μg of protein from each

region was used and membranes were simultaneously probed with two antibodies: AB1511, a rabbit polyclonal (1:5000 dilution, Chemicon, Temecula, CA) that recognizes both GAD65 and GAD67 and anti β-actin as above. After incubation with the primary antibodies overnight at 4°C, alkaline phosphatase-linked goat anti-mouse or anti-rabbit secondary antibodies (1:5,000 dilution, Sigma, St. Louis, MO) were applied and the membranes were subsequently incubated with CDP-Star (NEB, Beverly, MA) reagent according to the supplier's instructions. For quantification, membranes were exposed to X-ray film for 0.5 to 20 minutes and 600 dpi scans of these films were analyzed with AIDA software (Raytest, Wilmington, NC). GABARB and GAD levels were normalized to actin and a density ratio (GABARB/actin or GAD/actin) was calculated for each sample. Each set of western blots included a serial dilution of brain protein that was used to identify the exposures where the densities were in the linear range for actin and GABARB or GAD.

3. RESULTS

3.1 Taurine Acts As a $GABA_A$ Receptor Agonist

Taurine has been shown to play a role in neurotransmission, but taurine does not satisfy the criteria of a classical neurotransmitter. However, there is increasing evidence supporting a functional interaction between GABA, glycine, and taurine (Kuriyama and Hashimoto, 1998; El Idrissi and Trenkner, 2004). In this study we examined the functional consequences of the interaction of taurine with GABA receptors. Taurine has been shown to increase plasma membrane chloride conductances by affecting bicuculline-sensitive chloride channels (Wang *et al.,* 1998; del Olmo *et al.,* 2000; Mellor *et al.,* 2000) Taurine has also been shown to act as a partial agonist of $GABA_A$ receptors in synaptic membranes (Quinn and Harris, 1995). Here we show that taurine activates Cl^- influx through $GABA_A$ receptors in cerebellar granule cells *in vitro* (Fig. 1).

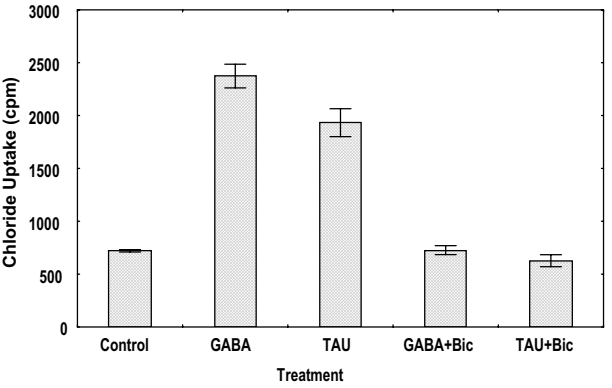

Figure 1. Taurine induces chloride uptake into cerebellar granule cells. Cells were treated with GABA (50 μM) or taurine (50 μM) and chloride uptake was initiated for 10 s. Both GABA and taurine induced a significant increase in chloride uptake. The fact that bicuculline (10 μM), a $GABA_A$ receptor antagonist, blocked taurine-induced chloride influx indicates the $GABA_A$ receptor as the site of taurine action.

The finding that taurine directly activates $GABA_A$ receptors *in vitro* suggests that taurine may interact with $GABA_A$ receptors *in vivo*. Consistent with this, we found that subcutaneous injections of taurine (43 mg/kg) to mice reduces kainic acid-induced seizure severity (El Idrissi, *et al.,* 2003). This suggests that the anti-convulsive effects of taurine, when injected before seizure induction, might be mediated by direct interaction with the $GABA_A$ receptors *in vivo.* We found that s.c. injection of taurine prior to seizure induction significantly reduced seizure severity, whereas long-term taurine intake though drinking water had an opposite effect, consistent with previous findings in rats (Eppler *et al.,* 1999). Mice chronically treated with taurine through supplementation in drinking water (0.05%) for 4 weeks showed increased susceptibility to KA-induced seizures, as demonstrated by a decreased latency for clonic seizures, an increased incidence and duration of tonic-clonic seizures, and a higher post-seizure mortality. Furthermore, supplementation of taurine resulted in significant biochemical alterations of the GABAergic system, mainly increased hippocampal GAD expression, reduced hippocampal $GABA_A$ receptor expression, and increased glutamate and GABA in the brain. We suggest that the effects of taurine are mediated through activation of $GABA_A$ receptors. The timing and duration of $GABA_A$ receptor activation determines when taurine has an inhibiting or promoting effects on seizure activity.

3.2. $GABA_A$ Receptor Expression in the Hippocampus of Taurine-Fed Mice

To investigate further biochemical alterations of the inhibitory system in taurine-fed mice, we analyzed $GABA_A$ receptor expression in the hippocampus from untreated controls and taurine-fed mice with a monoclonal antibody that recognized all β subunits of the $GABA_A$ receptor. The β subunits of the $GABA_A$ receptor is a key subunit that is present in virtually all of these receptors (Barnard *et al.,* 1998) and is thought to be required for receptor assembly and function (Conolly *et al.,* 1996; Homanics *et al.,* 1997). When we compared the expression levels in the hippocampi of taurine-fed mice and untreated controls, Western blot analysis revealed a reduction of β subunits expression (Fig. 2A). Expression of this subunit was reduced by approximately 40% (Fig. 2B).

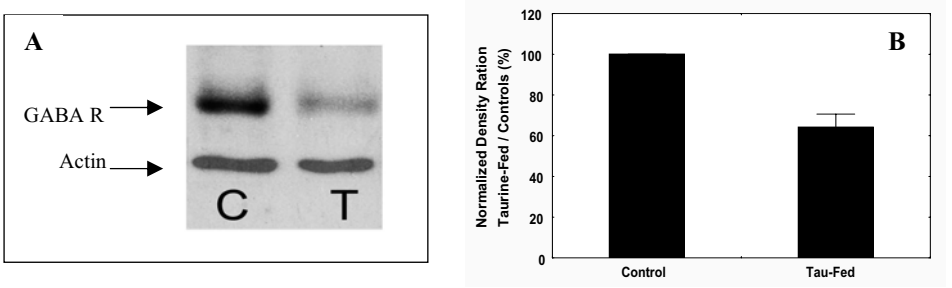

Figure 2. (A) Representative Western blot of $GABA_A$ receptor β, subunit and β actin expression in the hippocampus of (C) untreated control and (T) taurine-fed mice. All mice were tested at two months of age. Taurine was supplemented in water for four weeks before dissecting the brains. (A) Western blots were probed sequentially with a mouse monoclonal antibody that recognized the β, subunit of the $GABA_A$ receptor and a monoclonal antibody that recognized β actin. (B) Densitometry analysis showed that the expression level in taurine-fed mice is 65 ± 11 (mean ± SEM) as compared to untreated controls when normalized to actin expression. Four FVB/N controls and four littermates taurine-fed mice were analyzed in this study. Samples were obtained from the same hippocampi used to determine GAD expression (Fig. 3).

3.3. Taurine-Fed Mice Have Elevated Levels of Glutamate Decarboxylase

Analysis of amino acid levels in the brain of taurine-fed mice revealed elevated levels of GABA and glutamate (El Idrissi, *et al.,* 2003). Because glutamate is a precursor for GABA biosynthesis, this concomitant increase in the level of both neurotransmitters was accompanied by an increase in the expression of glutamate decarboxylase (GAD); the enzyme that catalyses the conversion of glutamate to GABA, as shown by Western blotting (Fig. 3). It is not clear at present if the increase in GABA levels in taurine-fed mice is a consequence of increased glutamate levels or it is the increased glutamate levels that lead to elevated GABA in this experimental model. Similarly, it is not known if the increased GAD levels are directs effects of taurine treatment of just a secondary adaptive mechanism to changes in the function of the inhibitory system in response to chronic treatment with taurine. The sequence of events leading to decreased $GABA_A$ receptor expression, and increased GABA, glutamate and GAD levels remain to be elucidated. However, these biochemical changes of the GABAergic system in taurine-fed mice is consistent with reduced threshold to KA-induced seizures.

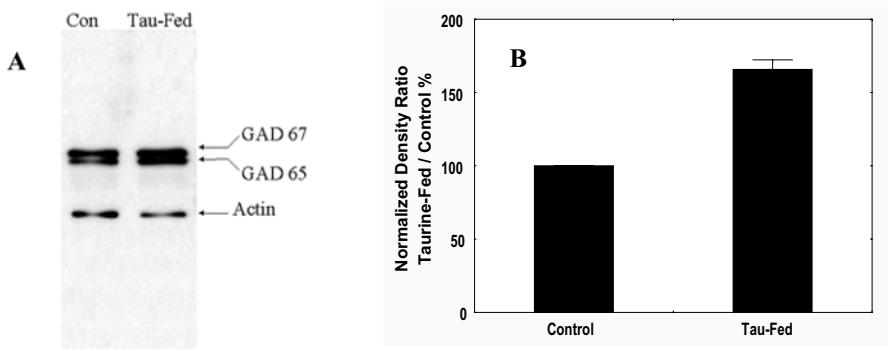

Figure 3. (A) Representative Western blot showing the expression levels of both isoforms of glutamate decarboxylase (GAD 65 & 67) in the hippocampus of untreated controls and taurine-fed mice at two months of age. Taurine was supplemented in the drinking water for four weeks before dissection. The blots were simultaneously probed with two antibodies against GAD and β actin. (B) Densitometry analysis showed that the expression level in taurine-fed mice is 166 ± 8 (mean ± SEM) as compared to controls when normalized to actin expression. Four FVB/N controls and four taurine-fed mice were analyzed in this study. The hippocampi were dissected from the same brains used for analysis the β subunit of $GABA_A$ receptors.

4. DISCUSSION

In the adult brain, inhibitory GABAergic interneurons modulate the activity of principal excitatory cells via their $GABA_A$ receptors, and thus adjust the excitatory output of neuronal circuits. In this study, we used a convulsive that induces limbic seizure and we used the threshold of seizure onset as a marker for the efficacy of the GABAergic system and brain excitability levels. Data of this study on the susceptibility to pharmacologically induced seizures showed that the excitability of limbic structures that subserve this type of seizures can be modulated through the interaction of taurine with the GABAergic system. Taurine activates chloride currents through $GABA_A$ receptors (Fig.

1). Injection of taurine (43 mg/kg) prior to seizure induction made the brain hypo-excitable and resistant to seizure induction. On the other hand, supplementation of taurine in drinking water (0.05 %) for 4 continuous weeks had the opposite effect than acute injection of taurine. We found that taurine-fed mice showed increased susceptibility to KA-induced seizures, as demonstrated by a decreased latency for the onset of clonic seizures, an increased incidence and duration of tonic-clonic seizures, increased neuronal death in the CA3 region of the hippocampus and a higher post-seizure mortality rate of the animals (El Idrissi *et al.,* 2003). Associated with this increased state of brain excitability, we found some biochemical changes in the GABAergic system. Chronic treatment with taurine in drinking water caused an increase in the levels of glutamate and GABA as well as the enzyme responsible for GABA synthesis, glutamate decarboxylase. We also found a reduced hippocampal expression of the β subunit of $GABA_A$ receptors.

GAD, which is responsible for GABA synthesis in GABAergic neurons, has two isoforms, 65 and 67 kDa (GAD65 and GAD67), encoded by different genes (Erlander *et al.,* 1991). The expression of both isoforms has been shown to be activity-dependent (Nishimura *et al.,* 2001; Ramirez and Gutierrez, 2001) and to be influenced by the effectiveness of GABAergic inhibition (Ribak *et al.,* 1988, 1993). Since reduced $GABA_A$ receptor expression would increase excitability, the increased GAD expression could be a compensatory mechanism for reduced efficacy of the inhibitory system. This is particularly interesting because increased GAD can be a compensatory response to the increased excitability (Ramirez and Gutierrez, 2001, El Idrissi, *et al.,* 2004) that would be the net result of decreased GABAergic inhibition.

The concomitant increase of glutamate with GABA in the brain of taurine-fed mice might also explain the reduced threshold for seizure induction. If there is more glutamate release following depolarization with kainic acid, it will lead to sustained activation of glutamate receptors resulting in high frequency discharge that typifies seizure activity. Alternatively, chronic taurine treatment may facilitate seizures through desensitization of the inhibitory system, primarily through $GABA_A$ receptors. Chronic elevated concentrations of taurine in the brain would lead to a desensitization of $GABA_A$ receptors or a down regulation of their expression. It has been established that $GABA_A$ receptors are involved in determining seizure threshold and are activated by taurine (Fig. 1). Consistent with this we found that chronic treatment with taurine resulted in a down regulation of the expression of the β subunit of the $GABA_A$ receptors. Therefore, taurine modulates seizure threshold through the interaction with $GABA_A$ receptors and chronic interaction may lead to functional modifications in the GABAergic system. Furthermore, we found that the taurine content of the brain was similar between taurine-fed and control mice (El Idrissi *et al.,* 2003). These findings indicate that taurine levels in the brain are highly regulated but might be differentially compartmentalized between intracellular, extracellular, neuronal and non-neuronal cells. We suggest that taurine-fed mice have elevated extracellular taurine levels, which would lead to sustained activation or at least binding to $GABA_A$ receptors. Such a chronic interaction of taurine with $GABA_A$ receptors may lead to down-regulation of $GABA_A$ receptor function or expression. In response to these changes, there is increased synthesis of GABA by GABAergic neurons, as compensatory mechanism to reduced post-synaptic inhibition. Consistent with this, the brains of taurine-fed mice have the highest GABA content, increased GAD expression and reduced expression of the β subunit of the $GABA_A$ receptors.

We suggest that activation of $GABA_A$ receptors by taurine prior to KA treatment causes neuronal hyperpolarization, which renders the brain more resistant to seizure induction. Whereas, sustained elevation of the extracellular levels of taurine exposes $GABA_A$ receptors to high levels of the agonist and might possibly have a desensitizing effect on these receptors, thus reducing the threshold for KA depolarization and seizure onset (Fig. 4). Alternatively, there may be increased glutamate release, because of its increased availability, and this leads to lower threshold for KA-induced seizures.

The findings of the current study reinforce the role of GABA inhibition in the maintenance of functional neuronal circuits characterized by a critical balance between excitatory and inhibitory inputs and suggest the potential complexity of changes in this system that result from environmental alterations. Therefore, hyper-excitability induced by chronic treatment with taurine results in complex biochemical changes that encompass key proteins involved in inhibitory synaptic transmission. Some of these changes in the GABAergic system seem to be compensatory for decreased GABAergic inhibition. It remains to be elucidated other possible modifications of inhibitory synapses that result from chronic taurine treatment such as GABA transporters and $GABA_B$ receptors.

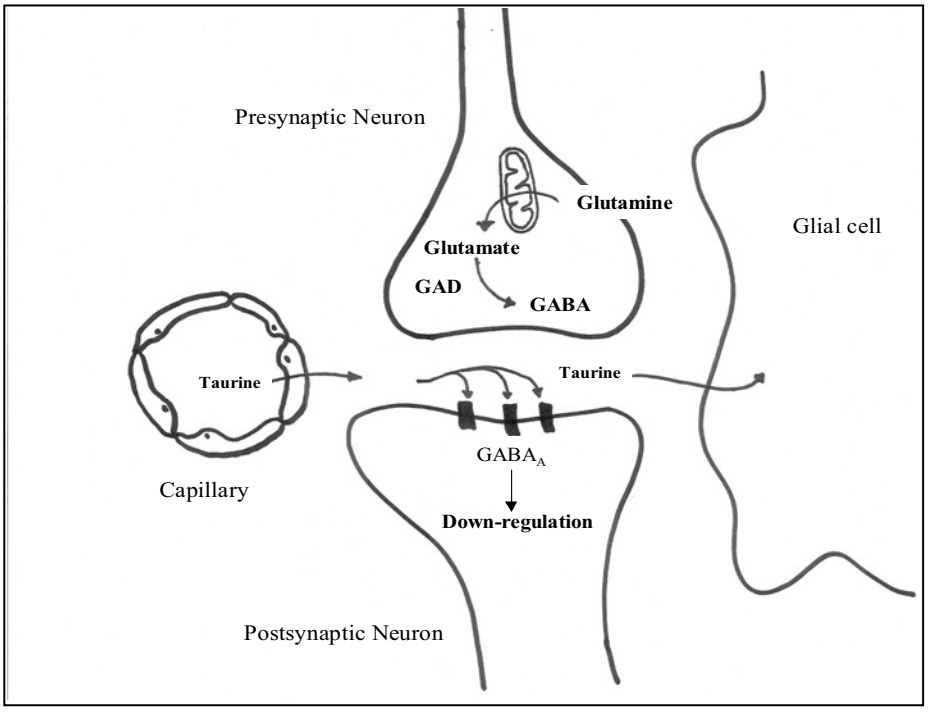

Figure 4. Proposed model for the possible interaction sites of taurine at an inhibitory synapse: chronic supplementation of taurine in drinking water results in a sustained interaction with $GABA_A$ receptors. This in turn results in a down-regulation of these receptors, which leads to reduced inhibition of post-synaptic membranes. As a compensatory mechanisms, pre-synaptic neurons up-regulate GAD expression to increase the efficacy of post-synaptic inhibition. This pre- to post-synaptic feed-back could be mediated through interneurons responsible for feed-backward inhibition and is accomplished to maintain appropriate excitability levels of neuronal circuits. The increase in GAD expression is accompanied by the elevated levels of GABA.

5. ACKNOWLEDGMENTS

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SYSTEMICALLY ADMINISTERED TAURINE

Part I. Central Nervous System Effects

Koho J. Miyamoto, Masumi R. Miyamoto, and Tadaomi A. Miyamoto*

1. INTRODUCTION

Oral taurine given chronically has been reported to decrease blood pressure in DOCA-salt hypertensive rats, and such effects were attributed to sympatholytic effects on the hypothalamic noradrenergic activity (Fujita *et al*., 1986). Anticonvulsant (van Gelder 1972; 1976; Beyer *et al*., 1988) and analgesic effects (Beyer *et al*., 1988) of taurine are known and suggest that the effects are not limited to the hypothalamus.

The well-known hypertensive response that plagues endotracheal intubation is an undesirable reflex response to the nociceptive pharyngeal manipulation with the laryngoscope mediated by the hyperactivity of the sympathetic division of the autonomic nervous system even in muscle-relaxant-paralyzed patients. To overcome it, either potentially dangerous deep anesthesia levels or alternatively light levels but supplemented with short acting ß-blockers or the combination of short acting α-blocking vasodilators and ß-blockers have been advocated.

We hypothesized that exogenous taurine intravenously given could effectively minimize such acute sympathetic reflex, and assessed the effects by (a) electroencephalographic (EEG) anesthesia depths, and (b) the blood pressure changes induced by pharyngeal stimulation, and glycemia responses to laparotomy and paraplegia-causing ischemia-reperfusion.

2. MATERIAL AND METHODS

2.1. EEG Anesthesia Depths

Six lightly anesthetized (isoflurane 0.5% + N_20 65%/O_2 30%) rabbits (2.7~3.0 kg body weight) were placed on the stereotactic table. Skull screw-electrodes for continuous

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Taurine 6
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EEG monitoring/recording and a brain cortex microdialysis probe were placed at the appropriate sites. After 3 stabilization periods of 22 minutes each, taurine (0.5 mmol/kg) was given by intravenous push every fourth period. The cortical dialysate (30 μl) of 4 periods (22 minutes each) were collected for each of the increments to a total cumulative dose of 2.0 mmol/kg for taurine determination. The anesthetic agent concentrations were kept unchanged. The EEG was recorded and digitized for Fourier transform analysis with a MacLAB (Australia) analogue/digital converter.

2.2. Blood Pressure and Glycemia Responses to Pharyngeal Stimulation

Thirty-two rabbits scheduled for spinal cord ischemia studies with or without taurine were assigned to this protocol; 21 were given taurine (3 mmol/kg as continuous drip of a 0.8 mol/l solution), and 25 ml of isotonic NaCl solution as placebo in 11. Both groups were infused over a period of 12 to 13 minutes.

The basic anesthesia, surgical preparation and methodology for inducing transabdominal spinal cord ischemia are detailed elsewhere (Miyamoto *et al*., 1998). Blind pharyngo-esophageal intubation (PEI) with a flexible 5 mm diameter thermistor probe bearing Foley bladder catheter in anesthetized rabbits ventilated via tracheotomy, provides a standardized reproducible model similar to but likely less reflexogenic than that encountered by anesthesiologists during rigid laryngoscopic endotracheal intubation. The blood pressures were recorded every 15 s after PEI for 3 min, then the rabbits were extubated and allowed to stabilize for 2 minutes. Intravenous drip of taurine or placebo was initiated, and the same PEI sequence repeated twice during the 11~12 minutes of infusion. The esophageal probe was kept in place after the last intubation for subsequent esophageal temperature measurements, followed by laparotomy.

In lieu of cardiac output measurements, expired [CO_2] was monitored (Respina, SANEI, Japan) while maintaining the unchanged rate of the respirator required to obtain 5% expired [CO_2] at baseline through the duration of the PEI study.

In addition to assessing blood pressure and glycemia, the response to laparotomy and ischemia-reperfusion was analyzed with the data collected from all animals whether given taurine (n=65) or not (n=107 for blood pressure; n=96 for glycemia) undergoing spinal cord ischemia studies.

2.3. Statistical Analyses

The commercially available software PRISM ver 3.0 (Graph Pad Software Inc, San Diego, CA. USA) was used. The values were expressed as mean ± SD but mean ± SEM bars are used in all figures for clarity purposes. Independent variables were compared using Mann-Whitney test, and/or one-way ANOVA with Tukey's multiple comparison test, or two-way ANOVA followed by Bonferroni post-test when comparing recovery courses. For continuous variables the paired Student t test and for correlation the linear regression was used. $P<0.05$ was considered significant.

3. RESULTS

3.1. Central Nervous System Effects of Systemically Administered Taurine

The taurine concentration in cortical dialysates increases logarithmically after systemic administration of taurine, reaching stable levels in 20 minutes (Fig. 1). The effects of taurine on the EEG are dose-dependent and time-dependent. Even though the concentrations of anesthetics were maintained unchanged to provide a light anesthesia level (I) through the entire study, taurine induced EEG changes suggestive of deepening of the anesthesia levels (II~V) in all 6 animals. EEG changes start after 15 to 20 minutes of each dose increment. Characteristically the high-amplitude (voltage), low-frequency waves of light anesthesia depth become low-amplitude, high-frequency ones as the cumulative taurine dose increases, and when 2.0 mmol/kg is reached, the EEG activity suggests anesthesia levels described as level V (Michenfelder, 1988). Cessation of electrical activity for long periods (burst suppression) occurring at level VI could not be induced even after further increasing the dose of taurine in a few pilot animals.

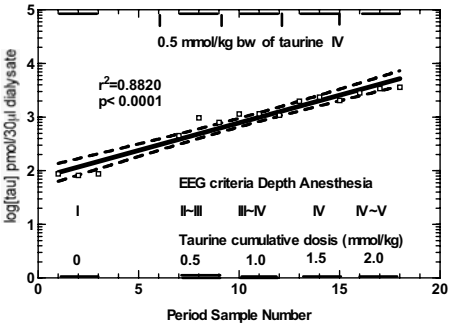


Figure 1. Systemically administered taurine dose-dependently increased logarithmically the cortical interstitial fluid taurine concentration, causing EEG changes suggestive of increasing anesthesia depths (from I to V) within 15 minutes of each dose administration. Abbreviations: bw=body weight; IV=intravenous.

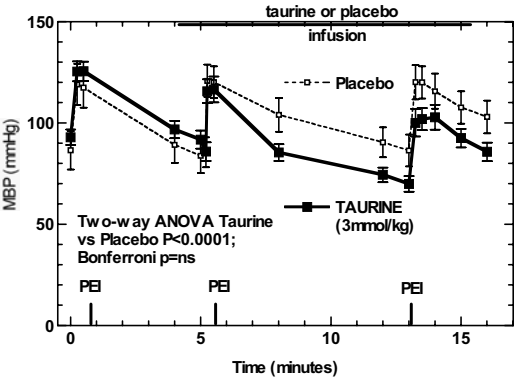
3.2. Sympatholytic Effects of Taurine

Taurine's hypotensive effects start shortly after the infusion is commenced but are clearly evident when 1 mmol/kg had been infused and are maximal after administration of 3 mmol/kg (Fig. 2A,B). The hypertensive response to pharyngeal stimulation could be minimized with 1.5 to 2.0 mmol/kg of taurine. Of interest is the lower blood pressure shortly after discontinuing anesthesia but still under its effects in a time frame unrelated to the neurological status in the animals given taurine (Fig. 3A).

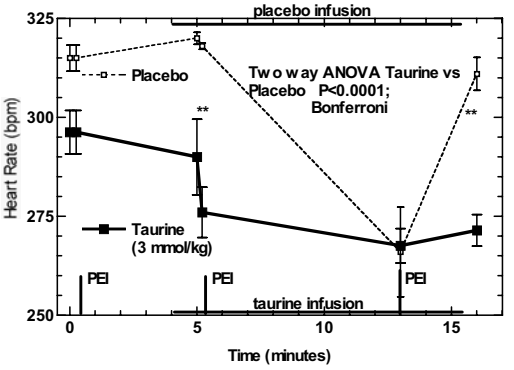
Bradycardic effects occur shortly after infusion is begun and are maximal by the time when 3.0 mmol/kg had been given (Fig. 2B).

The expired [CO_2] response is biphasic in the taurine group with an initial decrease during the first 1 to 2 minutes followed by a gradual return towards the baseline level,

A)



B)



albeit not complete over 10 to 11 minutes. In the placebo group, the [CO_2] falls with each pharyngeal stimulation and tends to remain abated (Fig. 2C).

The hyperglycemic response to laparotomy in the placebo animals is prevented with taurine; the glycemia concentration is even lower than at baseline (Figs. 2D and 3B).

4. DISCUSSION

Systemically administered taurine dose-dependently increased logarithmically the concentration of cortical interstitial fluid taurine, causing also dose-dependent progressive EEG changes that suggest increasing anesthesia depths within 15~20 minutes of each dose administration (Michenfelder, 1988, Fig. 1). Presumably this is the time required for taurine to cross the blood-brain barrier. EEG changes suggestive of anesthesia depth level V were obtained with 2.0 mmol/kg, but increasing the dose further did not increase the depth of anesthesia. Thus, it was felt that 2.5 to 3.0 mmol/kg would be the therapeutically effective dose.

Blind pharyngeal stimulation with a flexible catheter in a ventilated animal via tracheotomy might be significantly less reflexogenic than endotracheal intubation using a rigid laryngoscope under paralyzed non-ventilated conditions. Nevertheless, it provides a standardized, reproducible sympathetic stimulus, not subjected to the variability of the anesthesiologist's skill to perform endotracheal intubation.

C)

D)

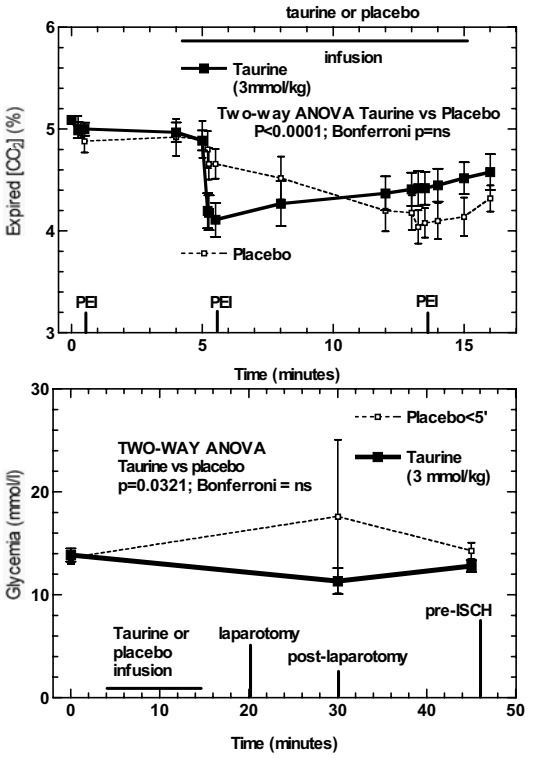
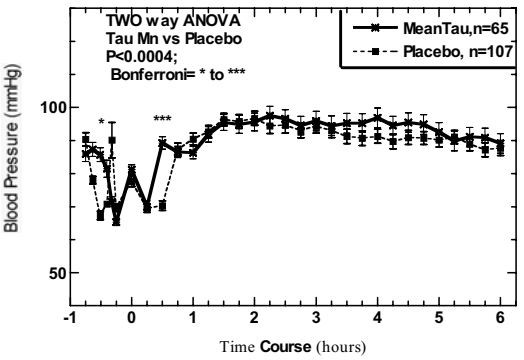


Figure 2. Taurine (3 mmol/kg body weight) induces (A) mild hypotension blunting the hypertensive response to pharyngo-esophageal stimulation (PEI) and (B) significant bradycardia. Taurine (3 mmol/kg body weight) restores (C) the expiratory [CO_2] and (D) prevents the hyperglycemic response to laparotomy. For clarity purposes the bars represent mean±SEM. Statistical significance of tests: * or $^{\#}$ P<0.05; ** or $^{\#\#}$ p<0.01; *** or $^{\#\#\#}$ p<0.0001 applicable to all figures.

The data clearly showed that taurine effectively blocks excessive cardiovascular and endocrine-metabolic sympathetic reflexes. Direct vasodilating effects of taurine are known (Busija and Leffler, 1989). The early blood pressure effects could be explained by this vascular dilatation but the decreased heart rate, especially that observed after 10 minutes, is more suggestive of central sympatholytic activity, which also explains the lower blood pressure shortly after terminating anesthesia, but before fully awakening.

The ventilatory rate was kept unchanged from that providing an expiratory [CO_2] of 5% at baseline. Based on Fick's principle, the [CO_2] changes were assumed to represent cardiac output changes. Whether or not the decrease of expired [CO_2] occurring in all animals concurrent to pharyngeal stimulation-induced hypertension prior to taurine represents a true fall in the cardiac output is speculative but suggestive. It is explainable on the basis of sympathetic vasoconstriction and concomitant decrease in the venous return. The biphasic expired [CO_2] in the taurine-given rabbits might indeed represent negative inotropy during the first three minutes followed by positive inotropy at ten min

A)



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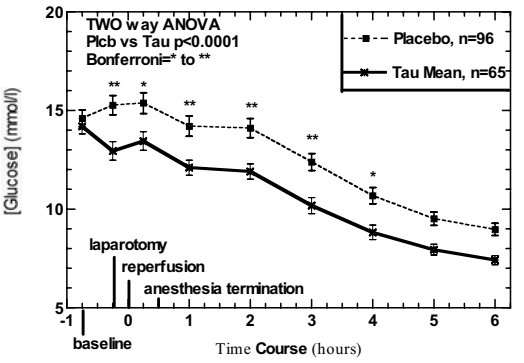

Figure 3. In taurine animals (A) the blood pressure at anesthesia termination is significantly higher than in placebo animals, minimally but lower than placebo during emergence from anesthesia and higher after anesthesia wears out. (B) Glycemia is significantly lower through the entire experiment. Moreover, the response to laparotomy is in opposite direction, increasing in placebo and decreasing in taurine animals.

(Sperelakis and Satoh, 1993). The time-dependent inotropic myocardial effects of taurine, in addition to the changing sympathetic tone that influences vascular resistance and venous return, might explain the trend to recovery of the expired [CO_2] in the taurine-given animals. In the placebo-given rabbits the progressive fall is suggestive of further vasoconstriction and declining of cardiac output.

The baseline glycemia levels of all rabbits were higher than those generally reported, and probably related to the stress of anesthesia induced in fully awake conditions without any pre-medication in our rabbits. Laparotomy increased glycemia regardless of the baseline levels in all placebo animals, which was uniformly blocked if taurine was given 15 to 20 minutes before laparotomy. This suggests that taurine sympatholysis contributes to hypoglycemia early after administration, in addition to the mechanisms described later.

The reported analgesic effects of taurine at the spinal cord level (Beyer *et al*., 1988; 1989; Hornfeldt *et al*., 1992) might have played a contributing role in controlling the postoperative glycemia. However, the glycemia levels in the placebo paraplegic animals were significantly higher than in those without paraplegia 60 to 90 minutes after anesthesia termination, in a time frame corresponding to that after the effects of anesthesia have worn out and supposedly enduring similar pain intensity. This suggests the stress of being paraplegic causes hyperglycemia, rather than the pain itself, that is, the

sympathetic tone plays a major role. Whether this hypoglycemic effect is only secondary to the sympatholytic activity of taurine and/or other mechanisms will be discussed later.

Thus, taurine solution started intravenously (2.5 to 3.0 mmol/kg body-weight) as a part of the pre-medication 15 to 20 minutes before anesthesia induction deepens anesthesia levels without increasing anesthetic concentrations. The sympatholytic effects may prevent the hypertension and hyperglycemia incidental to laryngoscopy and major surgical incisions. The sympatholytic effects might be beneficial for the conditions with excessive sympathetic tone, such as shock and extensive burns. Taurine administered to the organ donor before organ harvesting might be particularly useful in prevention of brain-death-induced cardiotoxicity of endogenous catecholamines (Farhat *et al*., 2001; Yeh *et al*., 2002), besides pre-conditioning the donor heart itself.

Part II. Systemically Administered Taurine Protects in Hypothermia and Normothermia

Tadaomi A. Miyamoto, Koho J. Miyamoto, and Masumi R. Miyamoto

1. INTRODUCTION

Although it has not been demonstrated in *in vivo* neural preparations, protective effects of taurine added to the suffusion fluid of hippocampal slices have been shown (Shurr *et al*., 1986). The hypothesis that systemic intravenous administration of exogenous taurine could be used as an innocuous supplement to enhance any central nervous system (CNS) protective strategy, including natural protective mechanisms, was tested in two models: (a) hypothermic rabbit's spinal cord ischemia lasting 60 minutes with or without taurine, and (b) normothermic ischemia lasting 2 to 5 minutes, also with or without taurine.

2. MATERIAL AND METHODS

2.1. Spinal Cord Ischemia

Spinal cord ischemia in rabbits is a well-established model (Herold *et al*., 1994; Ueno *et al*., 1994; Sakurai *et al*., 1998). Midline laparotomy and transperitoneal approach provides access for clamping of the aorta distal to the renal artery to induce spinal cord ischemia.

2.2. Hypothermic Ischemia

2.2.1 Surface-Induced Hypothermia and Rewarming

The detailed methodology is described elsewhere (Ohno *et al*., 1999). All animals were subjected to a 60-min period of aortic occlusion. Rabbits were supinely placed on a specially designed table equipped with a heat exchanging system, which allowed controlling esophageal temperature within ± 0.2 to $0.3^{o}C$ of the target.

The respiratory rate was controlled with a mechanical ventilator (SN-490-5-T2, Shinano, Tokyo, Japan) to maintain end-expiratory CO_2 concentrations of 4.5 to 5.0% (Respina IH26, San-ei, Tokyo, Japan) during normothermia. During hypothermia below $32^{o}C$ closer to 5.5-6.0% (equivalent to pH-stat management) maintained the blood pressure without the need of vasopressor agents. Surface cooling was induced with iced water containing bags placed on both the chest and abdomen after all neck surgical preparations were completed. The target temperature was maintained during the ischemic period by controlling the room and table temperatures. Rewarming by means of the heat-exchanging table, which excluded a 5.0 cm width of the paraspinal region, followed the release of the vascular clamps and abdominal closure. Anesthesia was terminated 90 minutes after reperfusion at the esophageal temperature of $35^{o}C$.

2.2.2. Neurologic Functional Assessment

The status of neurologic function was evaluated 360 minutes after reperfusion, or 270 minutes after anesthesia termination. For the purposes of this study, a simple assessment of whether the animals were totally recovered or protected (P+ able to keep the normal posture, to walk and to hop), or non-protected (P- if anything less than full recovery), was considered appropriate.

2.2.2a. Determining the Levels of Temperature Required to Protect From 60 Minutes of Ischemia with Taurine Supplementation. In group I or TAU P+ animals (n=5), the temperature was targeted to 30.5°C and taurine (10 mmol/kg body weight) administered by a drip; one third of it as soon as the venous line was available, one third during the cooling period, variable between 30 and 40 minutes, and the remaining one third 15 minutes before de-clamping the aorta.

2.2.2b. Determining the Levels of Temperature Required to Protect From 60 Minutes of Ischemia without Taurine. The animals with hypothermia alone were given lactated Ringer's solution in the equivalent volumes to that of taurine solution. The animals were randomly cooled to various levels of hypothermia (29°C to 30.1°C), which resulted in 2 subgroups with totally different neurological outcome (group II or Hypo P+, n=5 with full recovery and group III or Hypo P-, n=6 that failed to recover), but otherwise identically managed.

2.3. Normothermic Ischemia

Spinal cord ischemia was induced in 12 placebo groups randomly assigned to ischemia times differing by 0.75 to one minute between the groups, covering the range of 12~2 minutes at the rigorously controlled esophageal temperature of 38.3°C in 107 male Japanese white rabbits. Only the group of rabbits (placebo and taurine-given at the dose of 1.5, 2.0, 2.5, or 3 mmol/kg) subjected to ≦5 minutes ischemia were analyzed for this study. We mechanically maintained eucapnia (expiratory [CO_2] 4.5~5.0%) until anesthesia termination 30 minutes after re-perfusion (considered time 0), when switched to spontaneous air breathing.

3. RESULTS

3.1. Hypothermic Ischemia

All group I (taurine group) and II (hypothermia alone) rabbits recovered completely, but group I at 1.2°C higher temperature than group II ($p<0.0001$) and even 0.7°C higher than group III animals not given taurine ($p<0.0001$) that uniformly failed to recover neurological function (Fig. 4A).

3.2. Normothermic Ischemia

Taurine (3 mmol/kg) increased significantly the recovery rate (82% vs 36%, $p<0.001$; Fig. 4C) following 4 minutes of ischemia, the most critical ischemia time. Interestingly

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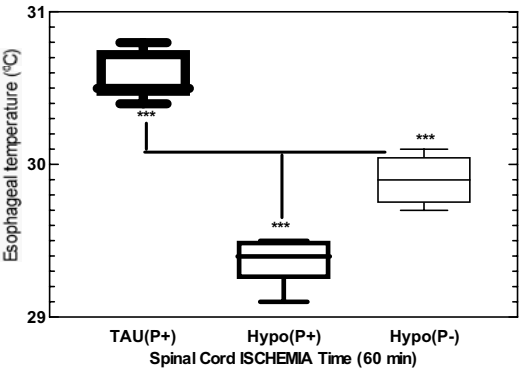

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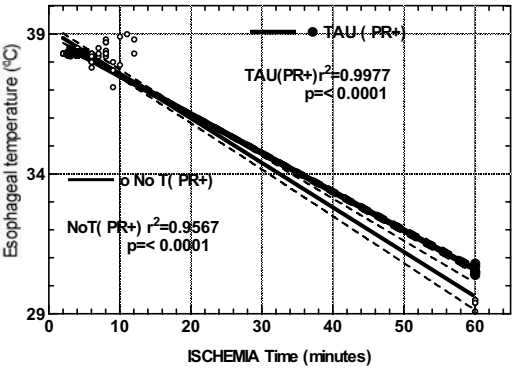
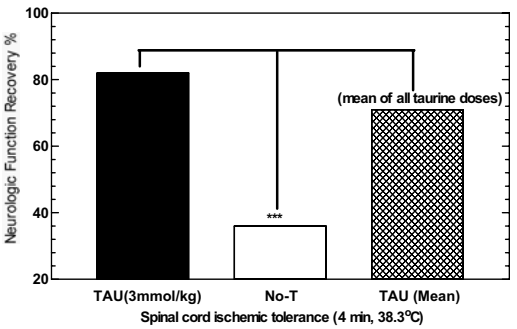


and paradoxically, larger doses are required to protect against 2.75- to 3-min than 4- to 5-min ischemia (Fig. 4D). Even though 1.5 mmol/kg could protect some of the animals subjected to 5-min ischemia, 2.5 mmol/kg seems to be the optimal dose to protect against 4-min ischemia, as lower doses will often fail.

4. DISCUSSION

Adenosine offers the possibilities of activating mainly A-1 receptors when given at small doses or mostly A-2 receptors when given at large doses (Fredholm, 1995). A-1 receptors decrease the release of ischemia-induced excitatory neurotransmitters by decreasing activity-evoked membrane depolarization (Schubert and Kreutzberg, 1993). A-2 receptors release taurine (Huxtable, 1989; Madelian *et al*., 1988; Miyamoto and Miyamoto, 1999), induce vasodilatation, and inhibit platelet aggregation, neutrophil activation and subsequent free radical production (Cronstein *et al*., 1990). However, when given systemically at doses to be neuroprotective, severe hypotension is induced,

C)



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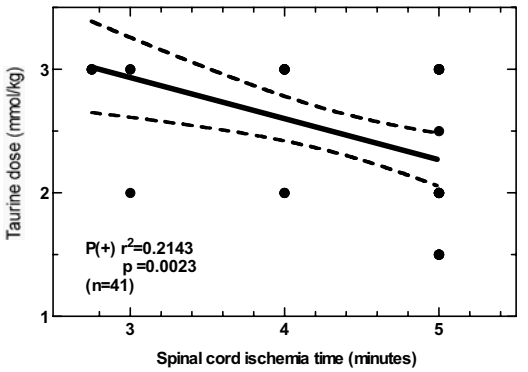

Figure 4. (A)Taurine protects 60 minutes of ischemia at 1.2°C higher than hypothermia alone and 0.7°C higher than the temperature hypothermia alone uniformly failed. (B) Taurine enhances the protective effects of hypothermia. Abbreviations: P+ or P- for protection (+ or -) with any used protective strategy; R+ for normothermic spontaneous recovery; T or TAU for taurine will be used through the entire chapter). (C). Taurine (TAU 2.5 to 3.0 mmol/kg) increases the normothermic ischemic tolerance to 4-min ischemia. (D) Optimal taurine dose to protect (P+) 2.75 to 5-min ischemia.

for which reason adenosinergic approaches have not met acceptance in general. Although taurine was found to have mild vasodilating effects at the dose used, the slight hypotension did not prevent the recovery of spinal cord functions. Moreover, higher pressures were kept after reperfusion and awakening from anesthesia than in placebo animals.

Our results confirmed the widely known protective effects of taurine *in vitro* (Shurr *et al*., 1986) in an *in vivo* hypothermic and normothermic spinal cord ischemia animal model, but we believe they could be extended to the entire CNS. Although observations are limited to a very small number, none of the fully recovered animals, 6 hours after reperfusion when observed the next day, had evidence of neurological worsening with or without taurine. This study does not address whether or not the late improvement could be expected to those not recovered in 6 hours, and whether or not late (3 to 4 days) neuronal death would be prevented in those recovered 6 hours post re-perfusion.

Apoptosis has been identified as the mechanism of delayed neuronal death in the gerbil hippocampus (Nitatori *et al*., 1995) and in the rabbit spinal cord (Sakurai *et al.,* 1998). However, late neuronal death following ischemic challenges within the studied

ranges may not occur if the animals are adequately protected in the acute phase with exogenous taurine. Taurine inhibits activation of p53, the apoptosis initiator gene (Takahashi *et al*., 2003); and prevents the ischemia-induced apoptosis in cultured neonatal rat cardiomyocytes inactivating the Akt/caspase-9 (Takatani *et al*., 2004).

Whether the doses of taurine similar to those used for normothermic animals would be equally protective when used to supplement hypothermia is not addressed by this study. Nevertheless, the contribution of taurine could be expressed in terms of the temperature difference between that required with taurine and that required by hypothermia alone to give protection at various ischemia times, as shown by their respective regression lines in Fig. 4B. In this study, albeit incomplete (since the upper temperature limits still compatible with the full recovery for both groups I and II were not completely mapped), the effect was 1.2^{o}C to protect against 60-min ischemia. This is a remarkable effect in the light of the fact that a temperature difference of only 0.5^{o}C sharply divided between the protected and non-protected animals by hypothermia alone. Cerebral ischemia-protective effects of hypothermia are secondary to reduction of oxygen-based free radicals (Zhao *et al*., 1996). Taurine might further reduce the production of free radicals (Wright *et al*., 1986; Huxtable, 1989), enhancing the protection afforded by hypothermia alone (Ohno *et al*., 1999).

The advantages of systemic administration of taurine in combination with only moderate hypothermia seem to be of practical value. Moderate hypothermia causes milder hematological effects than profound levels of hypothermia, while the protection of vital organs other than the central nervous system such as the heart (Milei *et al*., 1992), liver (Minor *et al*., 1995), and kidneys (Wingenfeld *et al*., 1996) are also enhanced by taurine.

We chose 4 minutes of ischemia at normothermia because it is worse than 5~9 minutes ischemia, and because taurine had to be administered post-reperfusion as well for significantly protecting >10 minutes ischemia (data not shown), which would complicate interpretation. By assessing 4-min ischemia, we postulate that the protective effects are mostly due to the administered taurine. Progressively more effective natural protective mechanisms seem to be recruited as the ischemia time increases. They become very effective with longer than 4~5-min ischemia (Blondeau *et al*., 2002). This explains the paradoxical need for larger doses of taurine to protect against shorter (2.75~3 minutes) than longer ischemia (4~5 minutes).

Although this study neither addresses the question of the mechanism how or when (during ischemia or re-perfusion), nor to which is the optimal dose or concentration needed for taurine to be protective when used in combination with hypothermia, our results confirmed that: (a) profound hypothermic levels are not absolutely essential to protect the spinal cord against 60 minutes of ischemia (29.4^{o}C) when ventilated under eucapnic conditions, (b) intravenous supplementation of taurine allows protection at such hypothermic levels (30.6^{o}C) when hypothermia alone does not (29.9^{o}C), and (c) systemically administered taurine also significantly increases the normothermic ischemic (critical time of 4 minutes) tolerance.

Part III. Systemically Administered Taurine: Pharmacologically Activated Mechanisms

Tadaomi A. Miyamoto, Koho J. Miyamoto, and Masumi R. Miyamoto

1. INTRODUCTION

As shown, sympatholytic, hypoglycemic and protective effects of intravenously given taurine (1.5 to 3.0 mmol/kg) are evident, but whether it only amplifies the already proven hyperpolarizing, anti-calcic, antioxidant (Wright *et al*., 1986; Huxtable, 1989), and antiapoptotic (Takahashi *et al*., 2003) direct effects or activates additional mechanisms is unknown. Taurine increases cell membrane sensitivity to insulin (Kulakowshi and Maturo, 1984). Oral supplementation of taurine in rats blunted age-related serum IGF-1declines (Carro *et al*., 2001). Because of the marked hypoglycemic effects observed in our early experience, we hypothesized that systemically administered taurine exerted its effects by acting upon the mechanisms determining the circulating insulin and/or IGF-1 levels.

2. MATERIAL AND METHODS

Male Japanese white rabbits (KBT-JW, 2.7 to 3.0 kg, n=111) were assigned to placebo-group (n=45 with 2~5 minutes of ischemia picked from the 107 placebo-group) and to taurine-group (n=65, 2~5 minutes ischemia time) to evaluate the normothermic protective effects of taurine. A freshly prepared solution of taurine (Sigma Chemical Co., St Louis, MO) was given intravenously at the dose of 1.5, 2.0, 2.5, or 3.0 mmol/kg; one third of the dose given every 5 minutes before laparotomy. The placebo animals received 3.75 ml of isotonic NaCl every 5 minutes, in total 11.25 ml, equivalent to the maximal volume the taurine animals received. Both groups were managed otherwise identically (Miyamoto *et al*., 1998; Ohno *et al*., 1999). Immediately after drawing blood specimens, their volume was replaced with isotonic NaCl, also used as maintenance of intravenous fluid (5 ml/kg/hour). After laparotomy closure, the trachea was repaired while maintaining anesthesia (isoflurane 1%) with a cone-mask. Anesthesia was terminated 30 minutes after aorta reperfusion (=time 0) in all rabbits. The neurological functions were assessed every 15 minutes after anesthesia termination with an objective neurological scoring (NS) from 0=under anesthesia to 7=able to hop (Miyamoto *et al*., 1998). The neurological outcome end-point was assessed 24 hours after the challenge as either fully (P+ for protection in taurine animals, R+ for recovery in placebo) regaining function or not. Less than the full functional return was considered non-recovery, which included a group of animals that returned to NS7 once but deteriorated later, termed P+/- or R+/-, and animals that never regained function, termed P-/- or R-/-, and combined as P(-) or R(-). Neurological function was assessed at 120 hours post-reperfusion. The aortic blood pressure was continuously monitored for the entire experiment duration (6 hours post-reperfusion), at the end of which all cannulae were removed. Digital read-outs were recorded every 15 minutes in all animals excepting one with an absent left carotid artery, which failed to be cannulated. Arterial blood (1.5 to 4 ml) was drawn at specific intervals

for hemoglobin, glucose, osmolality, and gas (9 specimens/animal) determinations made in the Hospital Laboratory facilities within 15 minutes of drawing. The blood specimens (5/animal) for taurine, γ-aminobutyric acid (GABA), insulin and insulin-like growth factor 1 (IGF-1) determination were centrifuged and the plasma samples frozen within 15 minutes of drawing. The frozen plasma was sent to an outside Laboratory (SRL, Tokyo, Japan) for determination of GABA (n=65) and taurine (n=46) by standard high-pressure liquid chromatography (HPLC) or immunoassay for insulin (n=39) and IGF-1 (n=15). Taurine and GABA were determined in 6 and IGF-1 in only 3 placebo animals.

3. RESULTS

The effects of taurine (Figs. 5~7, 9~13) could be divided in (1) early (within 30~60 minutes of administration, Table 1), (2) intermediate (1.0~3.75 hours post dosing), and (3) late (3.75 to 6.75 hours post administration).

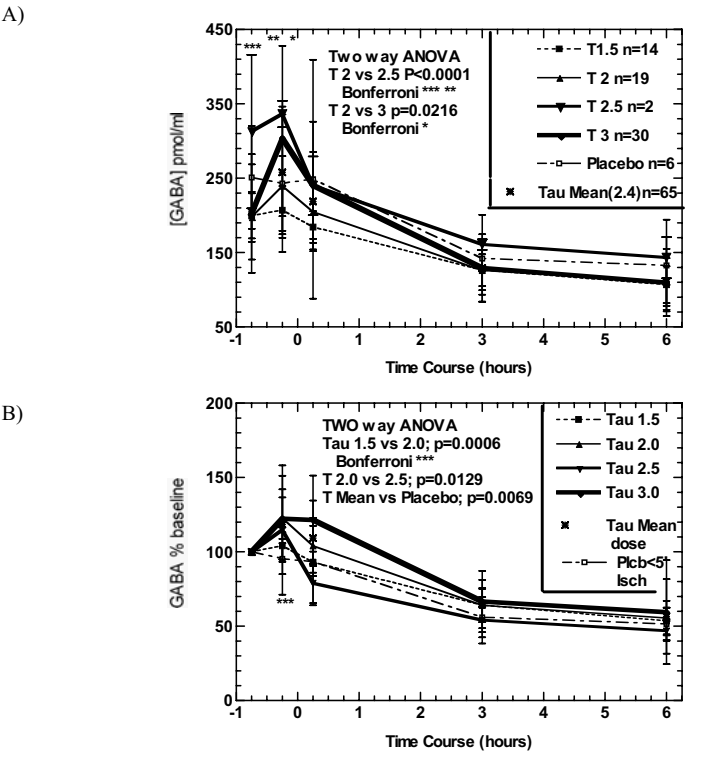

Figure 5. (A) Effects of 4 taurine doses on circulating GABA. (B) Effects expressed as % of baseline GABA concentration. The early effects are a dose-dependent increase, followed by an intermediate rapid decrease and late plateau of the blood levels.

3.1. Humoral Effects of Taurine

3.1.1. Taurine Effects on Circulating GABA

There was an early significant dose-related (2.5>3>2>1.5 mmol/kg) increase in GABA within 30 minutes of administration (Table 1). It was followed by intermediate effects consisting of significant and rapid dose-related decrease in GABA (2.5>1.5>2.0>3.0 mmol/kg) in the next 30 minutes, reaching the nadir at time 3=3.75 hours post-dosing to a slightly lower actual GABA concentration but still a higher percentage change from the baseline than in the placebo group (Fig. 5A,B). These relationships are maintained almost without change (plateau) to the end of the experiment.

3.1.2. Taurine Effects on Circulating Insulin

Early dose-related decreases in insulin are of smaller magnitude than those in IGF-1 but still significant within 30 minutes of dosing (Table 1), followed by an intermediate significant dose-related (3.0>2.0>1.5 mmol/kg) increase of % baseline at 3.75 hours post-dosing, and the late gradual return towards the baseline levels (Fig. 6A,B).

3.1.2a. GABA Changes Precede Insulin Changes. The early significant increase in GABA above the baseline levels precede the early decrease in insulin, which is followed by a dose-dependent decrease during the intermediate phase of 1 to 1.5 hours post-dosing before the dose-dependent insulin increase. The insulin increase reaches a maximum 3.75 hours post-dosing, coinciding with the lowest level of GABA (Fig. 6C).

Table 1. Early (30 minute) effects of 3 doses of taurine on plasma GABA, insulin, and IGF-1

Taurine dose (mmol/kg)	30 minute interval	GABA (pmol/ml)	Insulin (ng/ml)	IGF-1 (ng/ml)
1.5	Baseline	199.5 ± 30.31	0.291 ± 0.114	
	paired "t" P	0.189	0.64	NA
	Post-LAP	207.1 ± 32.37	0.273 ± 0.155	
2	Baseline	198.1 ± 33.75	0.429 ± 0.220	66.28 ± 19.67
	paired "t" P	P<0.0001	0.011	0.029
	Post-LAP	239.4 ± 40.4	0.271 ± 0.154	30.9 ± 8.981
3	Baseline	202.6 ± 79.9	0.193 ± 0.073	80.39 ± 31.5
	paired "t" P	0.001	0.007	0.001
	Post-LAP	242 ± 84.14	0.136 ± 0.074	46.26 ± 21.61
Mean dose	Baseline	204.7 ± 63.99	0.305 ± 0.179	73.39 ± 26.16
	paired "t" P	P<0.0001	0.002	P<0.0001
	Post-LAP	237.7 ± 67.43	0.223 ± 0.144	42.66 ± 18.59

The values are means±SD. The effects of 1.5 mmol/kg of taurine are not significant but 2 and 3 mmol/kg induce highly significant increases in GABA, while decreasing insulin and IGF-1 within 30 minutes of administration.

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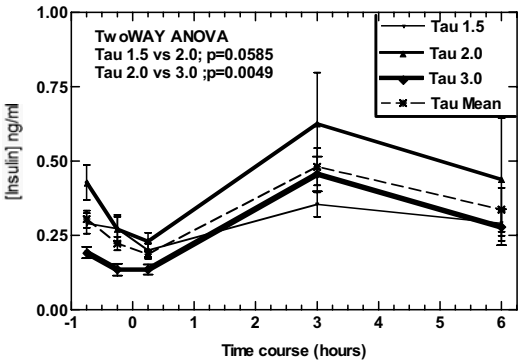

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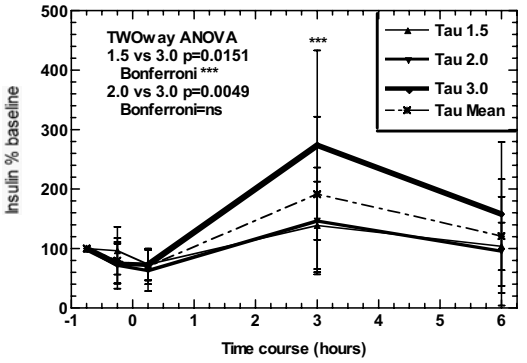

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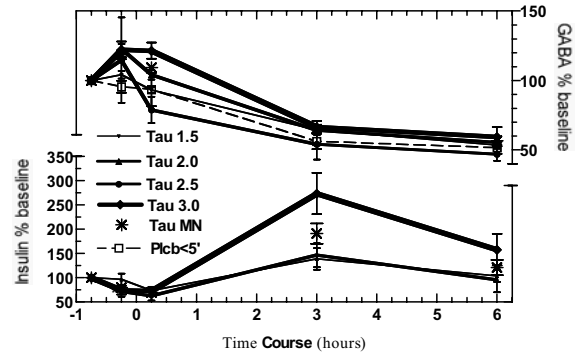


Figure 6. (A) Effects of 3 doses of taurine on circulating insulin concentration. (B) Expressed as % of baseline insulin concentration. Insulin was not determined in animals receiving 2.5 mmol/kg of taurine. GABA changes precede those of insulin.(C) Insulin changes are a mirror image of those of GABA and their respective peak and nadir occur 3.75 hours after taurine dosing (time 3.0).

3.1.3. Taurine Effects on Circulating IGF-1

Significant IGF-1 decrease (2.0>3.0>2.5 mmol/kg) occurs in 30 minutes (Fig. 7A,B). These decreases are of greater magnitude than those in insulin (Table 1). All taurine doses induce intermediate IGF-1 increase back to ≧baseline levels. Three mmol/kg reached almost the highest levels at 3.75 hours, but the dose of 2.5 mmol/kg at 6.75 hours

post-administration, although it was tested in only two animals. The above-baseline levels are recovered late with any of the doses; 2.5 mmol/kg being the most effective, it even curtails the initial decrease.

A)

B)

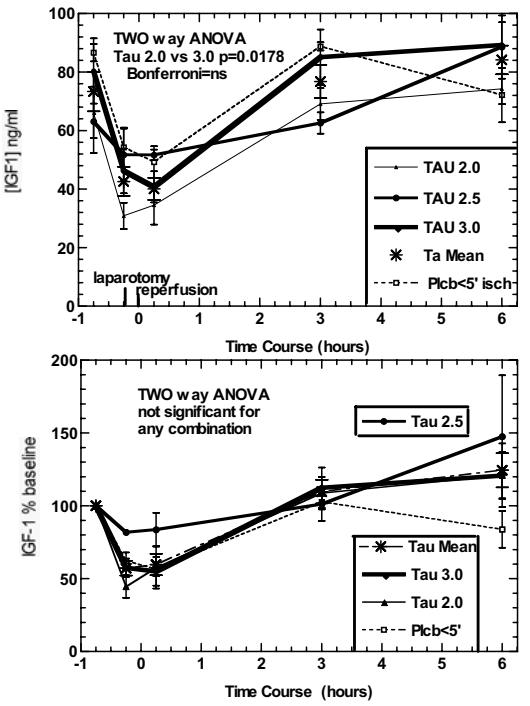


Figure 7. (A) Effects of taurine on circulating IGF-1 concentrations. (B) Effects expressed as % of baseline concentrations. Initially the IGF-1 concentration decreases but being followed by an intermediate and late increased levels. Maximal values are reached 6.75 hours post-dosing with all taurine doses, the 2.5 mmol/kg being seemingly the most effective.

3.2. Cardiovascular Effects of Taurine

Early prominent bradycardia and hypotension occurred when taurine concentration (Fig. 8A,B) and both the actual GABA concentration and the GABA levels relative to baseline (GABA % baseline) reached the maximum (as described earlier in the chapter) 20 to 30 minutes after administration (Figs. 5A,B, 9A,B,C). Taurine's dose-dependent cardiovascular effects were unequivocal ($p<0.0001$, two-way ANOVA) for all parameters even if Bonferroni post-test failed to show any significant difference. The lower blood pressure in the taurine animals during the first 2.75 hours of dosing seems to correlate mainly with the plasma taurine concentration (Fig. 8A,B) and secondarily with the higher GABA % of baseline (Fig. 9A). The taurine-induced transient but significant pressure increase 0.5 hours after reperfusion when anesthesia was terminated and the animals were allowed to spontaneously breath is interesting (time 0.5, Fig. 9A,B,C). The late effects correlate with the actual GABA concentration. After the full recovery from anesthesia

(time 2.0 on) the higher blood pressure correlates inversely with the lower actual GABA concentration rather than with the GABA % baseline (Fig. 9,B,C).

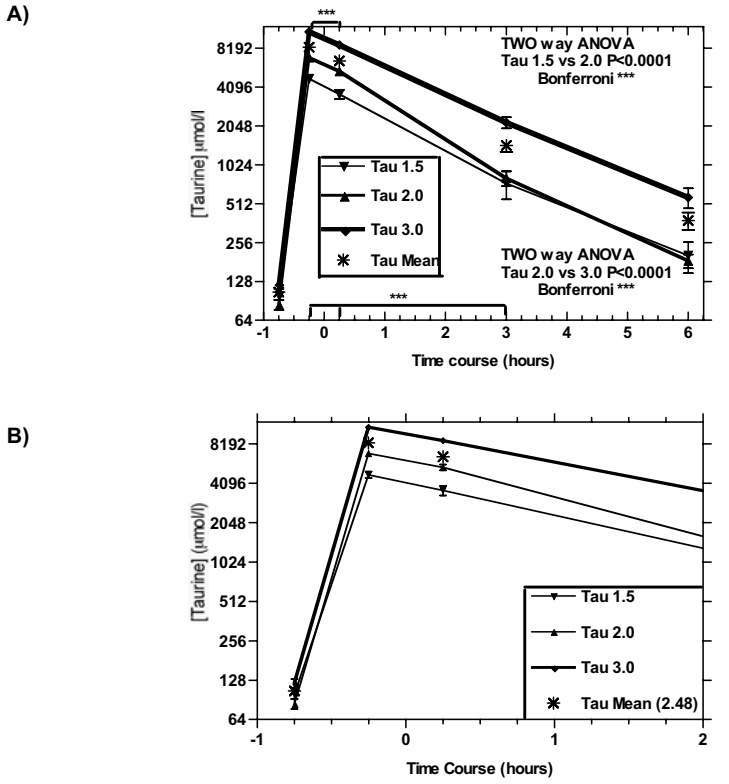


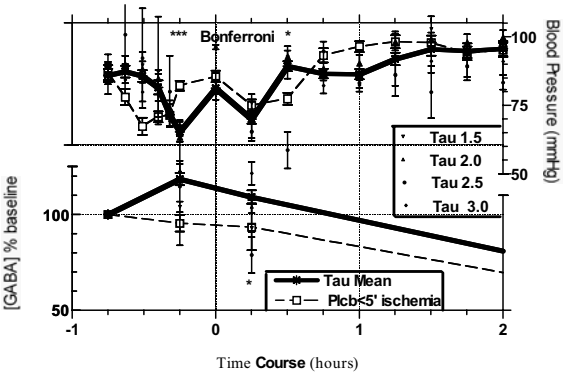
Figure 8. (A) Plasma taurine concentrations after 3 different doses of taurine. (B) Blow-out of the changes during the initial (sympatholytic phase) 2.75 hours post-dosing. Plasma taurine concentrations are dose-dependent, increasing by 80 to 100 times above the basal levels.

3.1. Hypoglycemic Effects of Taurine

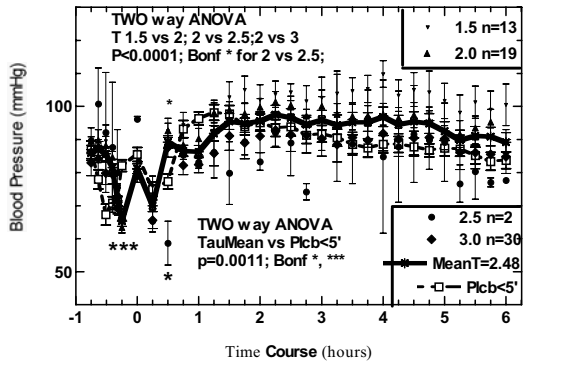
Early and intermediate significant hypoglycemic effects relative to the baseline values (%) compared to placebo are obvious through laparotomy, ischemia and during anesthesia recovery. They even persist to the late period, though differences were no longer significant. While the dose dependency of taurine effects on glycemia was clearly present ($p<0.0001$ two-way ANOVA), 2.5 mmol/kg being apparently the most effective dose, significant dose differences could not be observed (Fig. 10A,B).

3.3.1a. Temporal Correlation of Glycemia Changes With Those of IGF-1 and Insulin. The early hypoglycemic effects correlate mainly with the early IGF-1>insulin decrease, the intermediate effects with the significant insulin increase, and the late effects mainly with the increasing IGF-1 and to lesser extent with the still high insulin levels (Figs 6, 7 10B).

A)



B)



C)

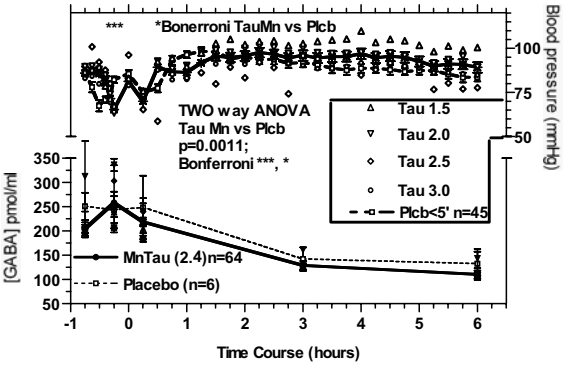


Figure 9. (A) Early and intermediate blood pressure changes correlate inversely with those of GABA % of baseline. (B) Effects of 4 taurine doses on blood pressure compared to the placebo group with ischemia <5 minutes (n=45). (C) Blood pressure in taurine animals is distinctly lower than in placebo animals immediately after anesthesia termination, but higher once the anesthesia wears out, coinciding with lower GABA than in placebo group.

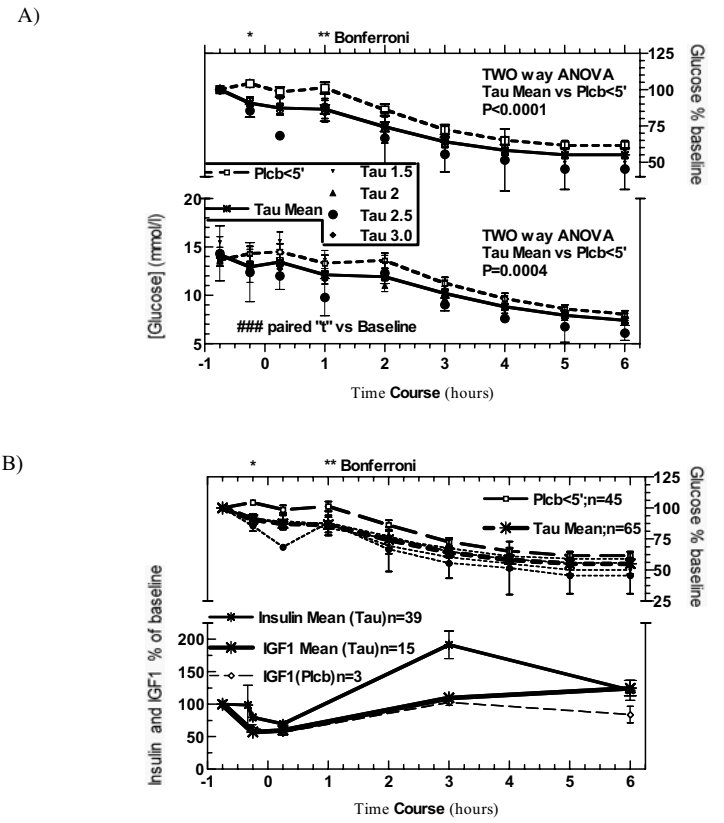


Figure 10. (A) Taurine effects on glycemia. Taurine induces significant hypoglycemia compared to placebo when normalized to baseline but there were no significant dose differences. (B) Hypoglycemic effects of taurine are secondary to effects on IGF-1 and insulin.

3.4. Effects of Taurine Relevant to Protection

3.4.1. Hypoglycemia

As already described, glucose concentrations decrease within 20 to 30 minutes of taurine administration (Fig. 2D), before the ischemic event, continuing for 6.75 hours (Fig. 10A). While the time course of glycemia in fully recovered animals with or without taurine was minimally different, the P(-) animals' glucose concentration was consistently lower. Likewise, the glucose % of baseline was significantly lower than in its placebo R(-) counterparts. In the taurine-given animals, the glycemia curves were practically identical regardless of the neurological outcome and blood pressure (Fig 11).

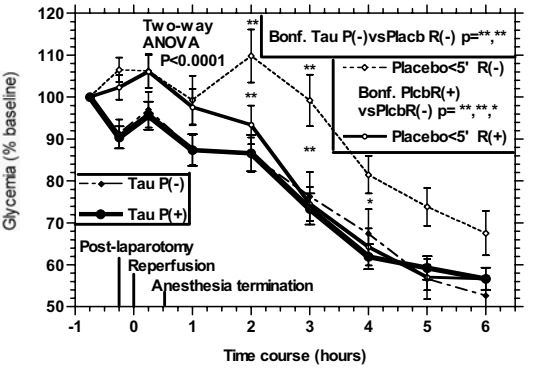


Figure 11. While glycemia of non-recovered placebo animals increases significantly 90 minutes after anesthesia termination, glycemia in taurine given animals is the same regardless of the neurological outcome.

A)

B)

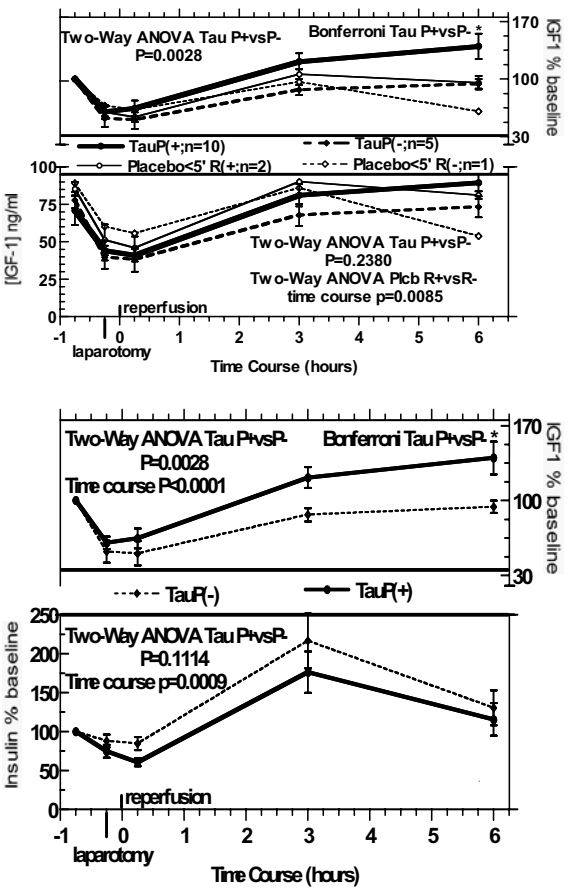


Figure 12. (A) Taurine-induced significant IGF-1 increases in P+ compared to P- animals at 6 hours post-reperfusion. (B) Insulin changes are P- >P+, though the differences were not significant.

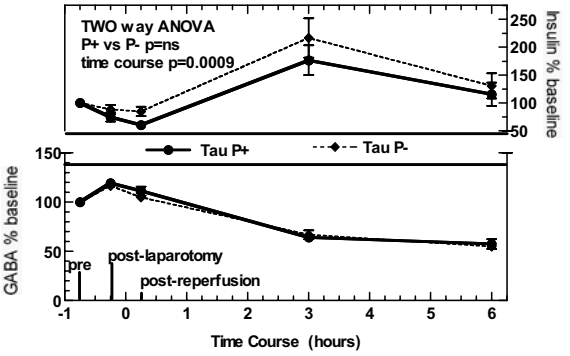


Figure 13. Although differences were not significant, the insulin changes of P-> P+ during the intermediate phase. However, the GABA changes in both groups are practically the same.

3.4.1a. IGF-1 Changes. The early IGF-1 decreases from the baseline of P(-)>P(+), though differences were not significant, are followed by an increase, peaking far above the baseline levels at 6.75 hours post-dosing in P(+), but barely recovering the baseline levels in P(-) animals, the differences being significant (P<0.05; Fig. 12A,B).

3.4.1b. Insulin Changes. The differences were not significant but the initial decrease is of a smaller magnitude, while the intermediate increase is greater in the non-protected than protected animals. Those in GABA are similar in both groups (Figs. 12B and 13).

4. DISCUSSION

4.1. Intravenously Administered Taurine Elicited Significant Central Nervous System Depression and Two Systemic Effects

4.1.1. Central Nervous System Depression

The well-known inhibitory effects of taurine (Huxtable, 1989) become apparent within 15~20 minutes of administration and the inhibition is dose-dependent. The functional correlate of such inhibition is deepening of the anesthesia level when assessed by EEG criteria. These inhibitory effects were obtained in conjunction with a basal anesthetic. Whether it would occur in the absence of anesthetics is not addressed but an equivalent anesthesia depth obtained without supplementary taurine requires increasing concentrations of anesthetics which might not be desirable for the side effects that may even be dangerous.

4.1.2. Sympathetic Inhibition, Blockade or Sympatholysis

The vasodilating effects of taurine (Busija et al., 1989; Huxtable, 1989) could explain the hypotension during its administration, but that observed 20~30 minutes post-dosing concurrent with bradycardia or during anesthesia emergence suggests sympathetic inhibition or blockade. Taurine injected intracerebro-ventricularly induces

bradycardia. Direct sympathetic inhibition and parasympathetic stimulation (Paakkari *et al*., 1982), and/or interaction with $GABA_{A\ and/or\ B}$ receptors of the spinal intermediolateral nucleus, the rostral ventrolateral area of the medulla, substantia nigra pars reticulata, hypothalamus, or amygdala which regulate sympathetic and cardiovascular systems have been proposed. Both the blood pressure and heart rate are decreased by microinjections of GABA or GABA agonists into those areas (Amano and Kubo, 1993; Avanzino *et al*., 1994; Madorin and Calaresu, 1994; Ye *et al*., 1997; Yoshida *et al*., 2002; Dampney *et al*., 2003). The role of taurine-induced activation of $GABA_{A\ and/or\ B}$ receptors is thus highly suggestive.

In our preliminary microdialysis HPLC studies using a carrier optimized for taurine, we confirmed that taurine freely crosses the blood-brain barrier, reaching various areas of the brain while GABA does not (Kakee *et al*., 2001). Thus, taurine was recognizable even at the baseline as a discrete peak, while GABA appears rather late and is broadly spread in the chromatogram and could not be assessed neither at the baseline nor after a significant increase in the circulating levels induced by intravenous taurine administration. Accordingly, we assume that the given taurine acted upon above brain sites rather than the circulating GABA.

Intravenously administered GABA-induced vasodilatation is particularly prominent in brain vessels, especially in the basilar artery. Whether these effects are mediated by the vascular smooth muscle cell $GABA_{A\ and\ B}$ receptors and/or presynaptic cells of the post-ganglionic sympathetic fibers is not clear but known (Fujiwara and Muramatsu, 1975; Starke and Weitzell, 1980; Anwar and Mason, 1982; Billingsley and Suria, 1982; Alborch *et al*., 1984; Lai *et al*., 1988; Minuk and MacCannell, 1988; Shirakawa *et al*., 1989). In agreement with those studies, the blood pressure decreased with increasing GABA levels. The transient but significantly higher blood pressure in the taurine animals 30 minutes after reperfusion when anesthesia was terminated and allowed to breath spontaneously, in spite of the pressures having been lower than in the placebo group through laparotomy and aortic clamping, suggests that they emerge from anesthesia with better cardiorespiratory reflexes and greater venous return.

We interpret that the increased tissue GABA uptake is the reason for the taurine-induced significant intermediate decrease in GABA. Though differences were not significant, the blood pressure response during anesthesia emergence is opposite when taurine had been given (lower) or not (higher). The lower blood pressure in the taurine group, in spite of the lower circulating GABA levels, is explainable by taurine-induced sedation, acting synergistically with those of residual anesthetics. The animals appeared well sedated during that time, concordant to the reported inhibition of gross motor behavior after intracerebroventricular injection of taurine (Frosini *et al*., 2003). After the full recovery from anesthesia, the lower blood pressure in the placebo group depends on the slightly higher circulating GABA concentration (Minuk and MacCannell, 1988). The higher pressure in the taurine group is desirable after surgery or ischemic events.

4.1.3. Hypoglycemic Effects

Anesthesia stabilization decreases the baseline hyperglycemia caused by the stress of anesthesia induction in all animals. However, the surgical stress of laparotomy increased glycemia in all animals. The hyperglycemic effect over the baseline levels was significant in the placebo animals, whereas in the taurine animals glycemia remained significantly lower. The time courses of the hypoglycemic effects of taurine are dose-dependent,

significantly different from those in the placebo group and apparently related to changes in the IGF-1 concentrations.

The time courses of circulating IGF-1 in the placebo and taurine animals are markedly different ($p<0.0001$, two-way ANOVA), decreasing significantly from the baseline levels during the stress of laparotomy in all animals, but being taurine>placebo. Thus, taurine facilitates the extraction from the plasma, that is, the consumption or tissue uptake of IGF-1 during the initial 30 minutes post administration. The dose-related greater/faster recovery of IGF-1 levels back to the baseline in 3.75 hours and above baseline in 6.75 hours after administration also suggests an increased release and/or production of IGF-1.

Kulakowshi and Maturo (1984) ascribed the physiologic hypoglycemic effects of taurine to the increased sensitivity to insulin at the cell membrane level rather than to the increase in insulin release. Accordingly, taurine administered systemically acts directly on cell membranes of the entire body or indirectly via the increased IGF-1 uptake (Venters *et al*., 1999), but either way the effect is expressed as an initial decrease in the blood level of insulin, i.e., increased insulin consumption or tissue uptake. The intermediate taurine-induced insulin release or production might be the result of the combined effect of taurine *per se* (Bustamante *et al*., 2001) and the dose-dependent taurine-induced GABA changes. Insulin secretagogue effects of GABA are known (Adeghate and Ponery, 2002; Brice *et al*., 2002).

Thus, we assume mechanistically that the early hypoglycemic effects of taurine are mediated by IGF-1>insulin uptake. The intermediate hypoglycemic effects might be secondary mainly to insulin>IGF-1 production relative to the baseline. The late (6.75 hours) hypoglycemic effect correlates with the late effects of taurine, causing continuing increase in IGF-1 rather than in insulin levels, which, though still higher, have decreased almost to the baseline levels (Figs. 5-7, 10B). Although only two animals were tested with the dose of 2.5 mmol/kg of taurine, it was the most effective dose to elicit both sympathetic blockade and hypoglycemic effects concordant with the largest GABA and IGF-1 responses. Furthermore, in the placebo animals IGF-1 decreased from time 3 to 6, though insulin changes are unknown.

4.1.4. Protective Effects of Systemically Administered Taurine

The lactic acidosis that glycemia >10~12 mmol/l induces is detrimental to CNS ischemia (Li *et al*., 1995). In addition to the hypoglycemic effects that should decrease lactic acidosis, CNS protective effects of GABA or GABA agonists (Nelson *et al*., 2000), IGF-1, and insulin (Shuaib *et al*., 1995; Wan *et al*., 1997; Kneussel, 2002) are known. Taurine induces changes in all three hypoglycemic factors: GABA, IGF-1, and insulin.

Although the availability of circulating IGF-1 could be increased by chronic exercise (Carro *et al*., 2001), increasing the IGF-1 concentration acutely within a few minutes could only be accomplished with administration of exogenous IGF-1 (Nakao *et al*., 2001). The effects of IGF-1, a pleiotropic polypeptide with a wide range of actions in the central nervous system and other tissues, may or may not be the major cause of the hypoglycemic effect. However, its biological significance for the protective actions might be far more important than its effects on glucose *per se*. Of the three hypoglycemic factors, insulin differed somewhat between the protected and non-protected animals, but IGF-1 was the only one with significant differences between the two groups. Some of the IGF-1 effects include: (a) attenuation of the up-regulation of nitric oxide synthase

expression (Sharma *et al*., 1998), (b) bi-directional regulation of the p38 kinase and c-Jun N-terminal protein kinase (Cheng and Feldman, 1998), (c) interaction with glycosaminoglycans (Gorio *et al*., 1998), (d) activation of the nuclear factor kappaB and phosphoinositol 3-kinase (Heck *et al*., 1999), (e) thyrosine phosphorylation of insulin receptor substrate-2 (Venters *et al*., 1999), which could explain the taurine-induced increased insulin sensitivity postulated by Kulakowshi and Maturo (1984) and the increased insulin uptake observed, (f) interaction with estrogen receptors (Azcoitia *et al*., 1999), (g) regulation of the Akt kinase (Kermer *et al*., 2000; Zheng *et al*., 2000; Nitta *et al*., 2004), (h) inhibition of caspase-3 (Kermer *et al*., 2000), and (i) increased expression of the antiapoptotic Bcl-xL protein and inhibited expression of the proapoptotic Bax protein (Zheng *et al*., 2000; Nakao *et al*., 2001).

Khan *et al*. (2000) and Shuaib (2003) did not observe neuroprotection by taurine delivered locally into the CA1 region of the gerbil hippocampus via previously stereotactically inserted microdialysis cannula (taurine being perfused at the dose of 25 mmol/kg/h) and subjected to 5 minutes of forebrain ischemia. Whether the dose actually delivered would be equivalent to the dose our rabbits' spinal cord received is only speculative. Most importantly, the delivery of taurine only to the CA1 region, regardless of the dose, bypasses the beneficial effects elicited by the systemic administration and might have accounted for the failure to observe protection in their model.

Thus, we postulate that the protective effects of systemically administered taurine result from the sum of both taurine's direct local effects as well as the described pharmacological activations. The direct effects are the known effects of taurine on cell membranes, which may also include the uptake of circulating IGF-1 as suggested by our results. The early decrease in the circulating levels of IGF-1 could be interpreted as amplification of tissue uptake of IGF-1. By increasing the uptake of already made circulating IGF-1, taurine administration enables all tissues avail of these multiple IGF-1-mediated protective actions effectively without delay. Of them, activation of the PI3k/Akt pathway and inhibition of caspase-3 are particularly relevant for protection. In ischemic tissues, the physiological effects of locally released endogenous taurine might be amplified by the exogenous taurine-induced IGF-1 uptake/production, in addition to the protective effects of exogenous taurine *per s*e, independently from IGF-1. The increase in circulating IGF-1 levels most likely results from pharmacological activation of tissue IGF-1 production, the liver being the most efficient organ. Thus, taurine and IGF-1 effects acting in concert lead to prevention of apoptosis (Kermer *et al*., 2000; Nakao *et al*., 2001; Nitta *et al*., 2004), which is characterized by delayed death after ischemia of the spinal cord or brain and many other tissues, thus amplifying ischemic pre-conditioning. Taurine increased the expression of anti-apoptotic protein Bcl-2 and decreased the cellular levels of p53, a putative initiator of apoptosis, in cardiomyocyte cultures rendered hypoxic (Takahashi *et al*., 2003). Whether these effects are directly elicited by taurine *per se* or mediated by IGF-1 synthesized by the cardiomyocytes is not clear. Although no pathological studies were performed, excepting one of the P(+) rabbits (1/41=2.4%) dosed with only 1.5 mmol/kg of taurine subjected to 5 minutes ischemia, none of the animals developed functional deterioration with several days delay, supporting the view that 2.5 mmol/kg might be the optimal dose to prevent effectively apoptosis occurring with ischemia of <5 minutes (Fig. 4D), in agreement with the maximal protection of cultured neurons at 2~3 mmol/l (Shurr *et al*., 1986).

In summary, to our knowledge no other single drug is known to increase the circulating levels of GABA, IGF-1, and insulin, i.e., production of all three coordinately

in tissues. Even though all three are involved in protection against ischemia, the changes in IGF-1 are especially important. In particular, intravenous taurine administration might provide a new emergency therapeutic approach to clinical conditions of the CNS. However, because the observed taurine-induced pharmacological responses are of systemic nature, involving all three (GABA, IGF-1, and insulin), the beneficial effects could be assumed not to be limited to the CNS and should be obtainable even in insulin-deficient patients, though being possibly of lesser magnitude. Excessive cardio-respiratory depression and/or hypoglycemia starts to occur with taurine doses >12 mmol/kg given within a short period (data not shown), but at the doses used in our experiments undesirable effects should not be observed, making cumbersome close-monitoring unnecessary.

4.2. Limitations and Significance of the Study

While blood pressure, glucose, and GABA, excepting one with the absent left carotid artery, was determined in all (n=65) and insulin in two thirds of the taurine animals, IGF-1 was measured only in 15/66. Nevertheless, the temporal and dose-dependent correlation of the taurine-induced changes in IGF-1 and insulin with the effects on glucose and the protective effects are beyond any doubt. Extension of the concepts to all taurine animals may therefore be justified. The dose of 2.5 mmol/kg was the most effective to obtain both sympathetic blockade and hypoglycemic actions, being suggestively the optimal dose, though tested only in two animals. In the placebo animals, GABA and taurine were determined in six, IGF-1 in three, and insulin in none.

The significance of the study is that the sympathetic blockade and glycemia control of subjects with excessive sympathetic excitation and whole body pre-conditioning could be innocuously and effectively obtained within a short period by manipulating endogenous GABA, IGF-1, and insulin levels with intravenously administered taurine.

5. ACKNOWLEDGMENTS

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EFFECTS OF TAURINE ON CEREBRAL BLOOD FLOW PERFUSION, CELL APOPTOSIS, AND INFARCT VOLUME IN ACUTE CEREBRAL ISCHEMIC RATS

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1. INTRODUCTION

Taurine (2-aminoethanesulfonic acid) is one of the most abundant free amino acids in the mammals. This simple non-proteinaceous sulfur-containing amino acid has been thought to be a vital nutrient for development of the immature brain and survival of neural cells (Saransaari and Oja, 2000). Recently, taurine has been shown to be involved in many important physiological functions such as maintaining the structural integrity of the membranes, regulating calcium binding and transport, and functioning as an osmolality regulator, neuromodulator, neurotransmitter, and neuroprotective agent against L-glutamate-induced neurotoxicity (Foos and Wu, 2002). In addition, taurine has been shown to protect neural cells from excitotoxicity and prevent harmful metabolic events evoked by cell-damaging condition such as ischemia or hypoxia and epilepsy (Saransaari and Oja, 2002). Previous studies implied that taurine might be a potential and potent agent against cerebral ischemia. To corroborate its beneficial effects and explore their possible mechanisms, we investigated the effects of taurine on acute cerebral ischemia with respect of cerebral blood flow perfusion, cell apoptosis, neurological deficits and infarct volume.

2. MATERIALS AND METHODS

Adult male Sprague-Dawley rats (250 g±10 g) were randomly divided into 5 groups: ischemia (n=12), ischemia+taurine 10 mg/kg (n=8), ischemia+taurine 40 mg/kg (n=12),

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ischemia+taurine 80 mg/kg (n=8), and sham groups (n=6). Taurine was dissolved in saline and given (i.p.) at 5 minutes of ischemia and 5 minutes of reperfusion.

Transient cerebral ischemia was produced by a modification of the method of Longa *et al*. (1989). Under anesthesia, a segment of 4-0 monofilament nylon suture was inserted into the origin of the right middle cerebral artery to occlude the blood flow. After 1 h of occlusion, complete reperfusion of ischemic area was allowed by withdrawal of the suture. In the sham-operation group, the same surgical procedures were carried out except for the artery occlusion. Changes in the cerebral blood flow (CBF) of the ischemic cortex were monitored continuously from pre-ischemia to the early stage of reperfusion with a Laser-Doppler flowmeter (LDF, PeiFlux5000, Sweden) to ensure the success of artery obstruction and the observe the dynamic change of the CBF.

Neurological deficits were evaluated at 24 hours of reperfusion with 0~7 grade method. A rat exhibited the symmetrical behavior as a normal animal in 0 grade and could only lie on the left side because of the loss of supporting capability in 7 grade.

After 24 hours of reperfusion, the rats were quickly sacrificed. Eight brains in each group were removed and sliced into 2.0-mm thick sections, and then incubated in 20 g/l TTC for 30 min at 37^{o}C to observe the infarct area. The area/volume of infarcts was measured as in Swanson *et al*. (1990). Four brains in each group (ischemia, sham, and ischemia+taurine 40 mg/kg) were perfused with 40 g/l paraformaldehyde and cut into 30 μm thick coronal sections in a cryostat. To detect *in situ* fragmentation of double DNA damage histochemical staining with TUNEL was performed. The expression of active subtype of caspase-3 protein (caspase-3 p20) was observed by immunohistochemical methods. Immunostaining for caspase-3 was performed with the avidin-biotin peroxidase (ABC) method. The expression of caspase-3 mRNA was observed by *in situ* hybridization. The brain slices were incubated in hybridization buffer with the casapase-3 mRNA DIG-labeling oligonucleotide probe.

Changes in cerebral flow were evaluated as percentages, comparing to the base flow in pre-ischemia. Grades of neurological deficit were analyzed with the grading method. The cerebral infarct volume was estimated as percentage of the whole cerebrum. The number of TUNEL positive cells was counted and expressed as percentages in five (x200) random MCA areas. The level of expression of caspase-3 mRNA and protein was analyzed with the gray value. The statistical comparisons between two groups were performed with the unpaired *t* test. Significance between groups was assigned at $p<0.01$.

3. RESULTS

After 1 hour of ischemia and 24 hours of reperfusion, the degree of neurological deficits were severe (average 6 grade), and the infarct areas were larger than 30% of the whole brain in the model group. The infarct areas were distributed mainly in the ipsilateral striatum and cerebral cortex. Taurine 10 mg/kg had no significant effect on either neurological deficit score or cerebral infarct volume. However, the degree of neurological deficiency in the taurine 40 mg/kg and 80 mg/kg groups was slighter than that in the model group. The infarct volume in the taurine 40 mg/kg and taurine 80 mg/kg groups was likewise smaller than that in the model group (Table 1).

Continuous CBF monitoring in the local cortex showed that taurine increases the local cerebral blood flow. When the middle cerebral artery was successfully obstructed the local blood flow decreased immediately to less than 20% in pre-ischemia and persisted at the lower perfusion level during the whole ischemia stage. The lowered flow continued to the early stage of reperfusion, owing to long and severely low perfusion during the ischemic stage. When taurine (40 mg/kg) was given i.p. at the ischemic stage, the blood perfusion of the ischemic cortex started to increase by 50%~100% of the ischemic level from 10 min onwards and fluctuated about 30 min at a level higher than the ischemic level (Fig. 1).

Table 1. Neurological deficit score and cerebral infarct volume

Group		Neurological deficit score	Cerebral infarct volume (v/v%)
Sham		0	0*
Model		6(5~7)	31.2%±4.5%
Taurine	10 mg/kg	5(4~6)	29.3±4.6%
	40 mg/kg	3(2~4)	12.3%±1.3%*
	80 mg/kg	3(2~4)	12.0%±3.0%*

*Cerebral infarct volume is presented as mean±SD. Significant differences *vs*. model□*t*-test, p<0.01).

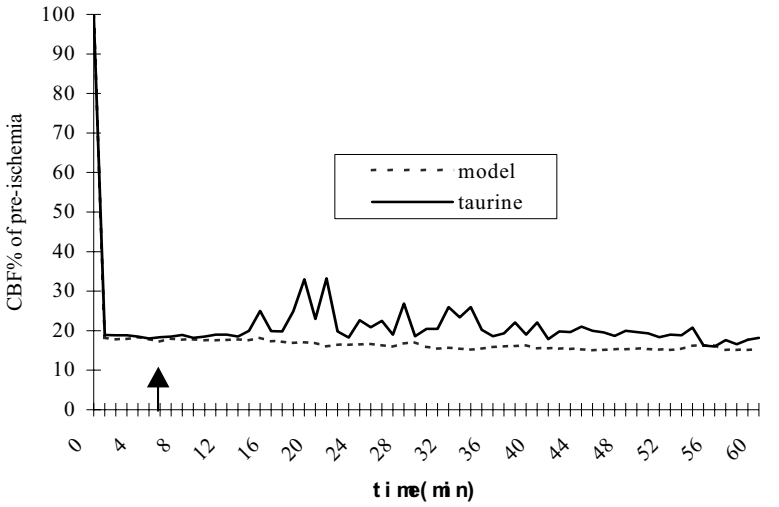

Figure 1. Effect of taurine on CBF in the local ischemic cortex. After taurine was given, the local blood flow increased from 10 min onward and fluctuated for 30 min (↑ dosing time).

We investigated effect of taurine on ischemic cell apoptosis. The results (Fig. 2) showed that there was a mild expression of caspase-3 mRNA and few immunoreactivities of caspase-3 p20 in the sham brain sections. When ischemia/reperfusion occurred, the expression of caspase-3 mRNA and immunoreactivities of caspase-3 p20 increased significantly. However in the ischemia+taurine group, the levels of expression of caspase-3 mRNA and caspase-3 p20 protein were lower. Similarly, TUNEL-positive cells were few in the sham group and increased in the model group, but were reduced in the ischemia+taurine group (Fig. 3). The decrease in the number of TUNNEL-positive cells and down-regulation of capase-3 mRNA and caspase-3 p20 protein in the taurine group imply that taurine is related to the anti-apoptotic pathways after ischemia and reperfusion.

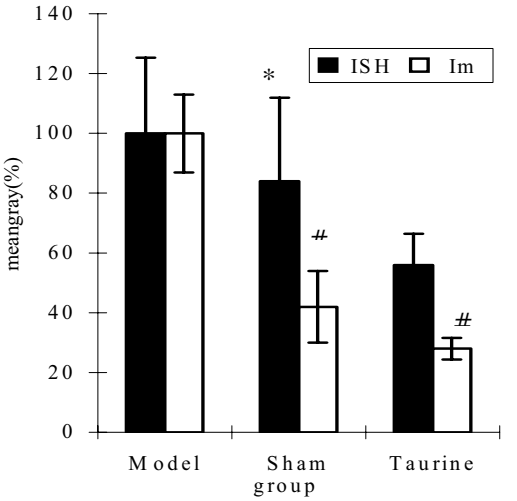

Figure 2. Effect of taurine on the expression of caspase-3 mRNA and caspase-3 p20 (mean gray% *vs*. model as 100%). *p<0.05, #p<0.01.

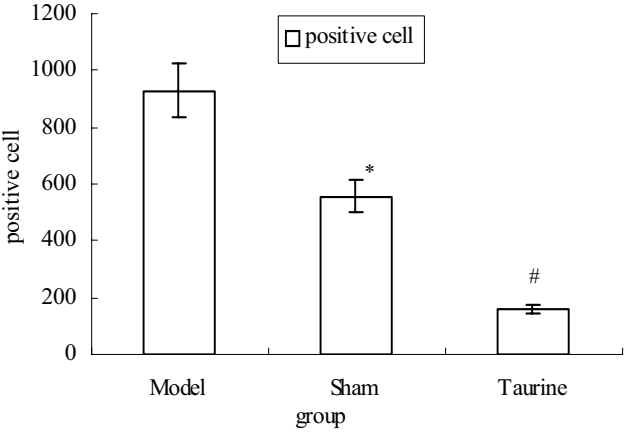

Figure 3. Amount of TUNEL-positive cells in the model and taurine groups (*vs*. model *p<0.05, #p<0.01).

4. DISCUSSION

In previous studies, elevated exteracellular levels of taurine were seen to enhance adenosine release in ischemia and contribute to the maintenance of homeostasis (Saransaari and Oja, 2003). Taurine could also be modified by NO (Saransaari and Oja, 2002) or electroacupuncture (Guo *et al.,* 2002). On the other hand, studies of Shuaib (2003) did not reveal neuronal protection of taurine. However, the results from our present research showed the protection of exogenous taurine by alleviating the neurological deficits and infarct volume.

Reduction of local cerebral blood flow is crucial to origin and development of ischemic cerebral injury. Our research demonstrates the CBF-increasing effect of taurine. We think that it is an important pathway of taurine to protect against cerebral ischemia by ameliorating microcirculation.

Apoptosis are likely to be involved in primary and secondary cell death in cerebral ischemia. Previous research reported that increased pro-caspase-3 immunoreactivity is seen in the penumbra following focal ischemia in the adult rats (Isidro and Planas, 2003). Recent studies provide direct evidences for distribution of caspase-3 activation following cerebral ischemia (Rami, 2003). Our present study showed that the expression of caspase-3 mRNA and caspase-3 (p20) protein and the number of TUENEL-positive cells increased in acute ischemic rats but decreased with taurine treatment. It suggests that taurine is involved in the cellular and molecular mechanism of anti-apoptosis process.

In summary, the results of our present studies showed that taurine could protect the cerebrum against acute cerebral ischemia by either increasing cerebral blood perfusion or suppression of cell apoptosis. It implies that taurine may be a potential and potent agent for cerebral ischemia therapy. However, further studies are needed to clarify how taurine affects cerebral blood flow and cell apoptosis.

5. ACKNOWLEDGMENT

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THE MECHANISMS OF TAURINE'S PROTECTIVE ACTION AGAINST ACUTE GUANIDINO NEUROTOXICITY

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1. INTRODUCTION

Chronic renal failure (uraemia) is characterized by a variety of CNS disturbances (uraemic encephalopathy) (Fraser and Arieff, 1988; De Deyn *et al.,* 1992; Moe and Sprague, 1994) and significant increases in plasma levels of a wide range of potential neurotoxins (Vanholder *et al.,* 2001, 2003). These toxins include guanidino compounds, increased brain and plasma levels of which have been reported in uraemic patients (De Deyn and Macdonald, 1990; De Deyn *et al.,* 1995; Tanaka *et al.,* 1999), and their neuropharmacological action has been examined using electrophysiological and binding techniques (De Deyn *et al.,* 2001, 2003; De Hooge *et al.,* 2003).

Taurine is known to be released from cells under a variety of cell damaging conditions (Saransaari and Oja, 2000) and to exert a cytoprotective effect., the exact mode of which is still uncertain (Schaffer *et al.,* 2003). Oxidative stress is known to occur during both acute (Himmelfarb *et al.,* 2004) and chronic renal failure (Himmelfarb and Hakim, 2003; Vaziri 2004) with probable involvement of lipid peroxidation (Vaziri, 2003; Prakash *et al.,* 2004).

The present study examines the effect of taurine and other antioxidants in ameliorating swelling by cells in incubated rat cerebrocortical minislices acutely exposed to a range of guanidino compounds, it being assumed that swelling in isosmolal media provides *prima facie* evidence of cellular dysfunction. Some of the findings have been published as an abstract (Law, 2005).

2. MATERIALS AND METHODS

The preparation of minislices, the incubatory medium, the calculation of cell volumes from the equilibrium distribution of [^{14}C]inulin within slices, and the statistical

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methodology, have all previously been described in detail (Zielińska *et al.,* 2003). In the present study incubations were performed for 60 min at 37°C. Media were supplemented with 5% (w/v) polyethylene glycol 4000 in order to prevent spontaneous swelling (Banay-Schwartz *et al.,* 1974; Law, 1994a). Additions to the incubation media were as follows, separately or in combination, as specified in the relevant text.

2.1. Guanidino Compounds

Guanidine hydrochloride (GH, 3 μmol/l), guanidinosuccinic acid (GSH, 40 μmol/l), α-*N*-acetylarginine (1.5 μmol/l), methylguanidine (7 μmol/l), N^G,N^G-dimethylarginine (ADMA, 1 μmol/l). Concentrations approximate to those reported in the plasma of uraemic patients (De Deyn *et al.,* 1995; MacAllister *et al.,* 1996).

2.2. Antioxidants

Taurine (1-20 mmol/l; unless stated otherwise 20 mmol/l was used), ascorbic acid (0.4 mmol/l), butylated hydroxytoluene (0.5 mmol/l), the free-radical scavenger *N*-acetyl-L-cysteine (10 mmol/l) and the lipid peroxidase inhibitor desmethyl tirilazad (U-74389G, BIOMOL Research Laboratories Inc., Plymouth Meeting, PA). In order to effect cellular uptake this was dissolved in DMSO at 50 mmol/l and added to incubation medium containing 3% (w/v) essentially fatty acid-free bovine serum albumin to yield a final concentration of 100 μmol/l.

2.3. Other Additives

The taurine transport inhibitor guanidinoethanesulphonate (GES, 1 or 10 mmol/l) (a generous gift from Professor S. S. Oja, University of Tampere), mannitol (20 mmol/l), the nitric oxide synthetase (NOS) inhibitor *Nω*-nitro-L-arginine (NNA, 0.5 mmol/l) and the $GABA_A$ receptor antagonist bicuculline (100 μmol/l).

Butylated hydroxytoluene and bicuculline were added at 1:1000 from a stock solution in DMSO. Unless otherwise stated, all reagents were obtained from Sigma Chemical Co. Because the largest degree of swelling was obtained with guanidinesuccinic acid (GSA, see below) the majority of experiments were performed using this compound, with significant results confirmed using GH.

3. RESULTS

Equilibrium cell volumes in the presence of guanidine compounds are shown in Table 1. Of the 5 compounds tested, all except ADMA caused swelling that was significant in comparison with the control value at the level $P<0.005$ or better.

The effects on equilibrium cell volume of various antioxidants, including variable concentration of taurine, in media containing GSA, are shown in Fig. 1. The solid and dashed lines shown, respectively, control volumes are volumes in the presence of GSA alone (from Table 1). The findings may be summarized as follows. (1) Taurine alleviated the swelling effect of GSA in a dose-dependent manner, swelling being completely abolished at 20 mmol/l. (2) Equimolar mannitol did not significantly affect GSA-dependent swelling; these observations were repeated using GH-media. (3) There were

no significant increases in volume due to GSA in the presence of ascorbic acid, butylated hydroxytoluene, *N*-acetyl-L-cysteine, desmethyl tirilazad or NNA. These findings were repeated using GH-media.

Table 1. Mean cell volumes under control conditions and in the presence of guanidino compounds

	Cell Volume (µl/mg dry wt)
Control	2.67±0.05
Guanidine HCl (GH, 3µM)	3.06±0.07***
Guanidinosuccinic acid (GSH, 40µM)	3.31±0.05***
N-acetyl arginine (1.5µM)	3.01±0.04**
Methylguanidine (7µM)	3.04±0.07***
N^G,N^G-dimethylarginine (ADMA, 1µM)	2.81±0.07$^{n.s.}$

The significance of guanidino-dependent swelling is shown as $^{**}P<0.005$, $^{***}P<0.001$. n.s. = not significant (n= 16).

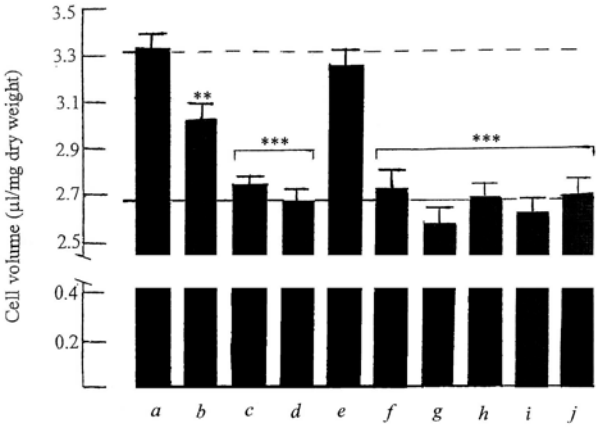

Figure 1. Mean cell volumes in the presence of GSA plus taurine (1-20 mmol/l, columns *a* – *d*), mannitol (20 mmol/l, *e)*, ascorbic acid (0.4 mmol/l, *f*), butylated hydroxytoluene (0.5 mmol/l, *g*), *N*-acetyl-L-cysteine (10 mmol/l, *h*), desmethyl tirilazad (100 µmol/l, *i*), Nω-nitro-L-arginine (0.5 mmol/l, *j*). The significances of the extent to which these additives reduced swelling by comparison with volumes in the presence of GSA alone are represented as $^{**}P<0.005$, $^{***}P<0.001$ (n=8 for concentrations of taurine < 20 mmol/l; otherwise 16). The solid and dashed horizontal lines represent, respectively, mean volumes under control conditions and in the presence of GSA (from Table 1).

Fig. 2 shows the results of incubatory variations that impaired or abolished the effects of taurine on GSA-dependent swelling. In the presence of taurine, GES (1 mmol/l) led to a ~50% reduction of the swelling caused by GSA alone, but cells remained

significantly swollen by comparison with control values $P<0.005$). Increasing GES to 10 mmol/l was without further effect. The remaining histograms show the results of treatment with the $GABA_A$ receptor antagonist bicuculline. Bicuculline alone was without effect, but reduced the ameliorative effect of taurine by ~40% ($P<0.005$). When GES and bicuculline were both present the effect of taurine was completely abolished. Cell volumes were significantly greater than in the presence of GES alone ($P<0.01$) or bicuculline alone ($P<0.025$). Comparable results were obtained using GH.

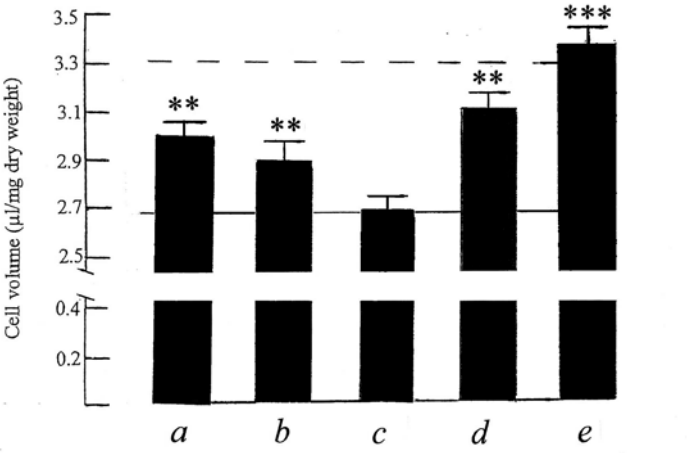

Figure 2. Mean cell volumes in the presence of GSA plus GES (1 mmol/l, column *a*, and 10 mmol/l, *b),* bicuculline (100 µmol/l, *c*), bicuculline and taurine (20 mmol/l, *d*) and bicuculline, GES (1 mmol/l) and taurine, *e*). The significance of swelling by comparison with volumes in the presence of taurine alone are represented as $^{**}P<0.005$, $^{***}P<0.001$ (n=16). The solid and dashed lines represent, respectively, volumes in the presence of taurine plus GSA (from Figure 1) and GSA alone (from Table 1).

4. DISCUSSION

The present study clearly shows that brain cells swell when acutely exposed to guanidino compounds. But it is not intended to imply that CNS dysfunction during uraemia *in vivo* can be attributed to oedema *per se*. Brain cells possess mechanisms enabling them accurately to regulate their volumes during long-term chemical or osmotic challenge (Gullans and Verbalis, 1993; Law, 1994b; Pasantes-Morales, 1996; Pasantes-Morales and Schousboe, 1997), but it may reasonably be inferred that cellular dysfunction will continue for as long as circulating neurotoxins are present.

The protective effects of endogenous taurine under brain cell damaging conditions are well established (Saransaari and Oja, 1998, 2000) and at least one component of its action appears to depend on its anti-oxidant properties (Schaffer *et al.,* 2003). In the present study exogenous taurine ameliorated guanidino-dependent swelling in a dose-dependent manner (Fig. 1, *a–d*). Since equimolar mannitol did not protect against swelling (Fig. 1, *e*) this effect may be viewed as molecule-specific rather than osmotic. Several other anti-oxidants were equally effective in abolishing swelling (Fig. 1, *f–i*).

The protective effect of ascorbic acid against spontaneous swelling has previously been demonstrated (Pasantes-Morales, 1996; Brahma *et al.,* 2000). The abolition of swelling by desmethyl tirilazad suggests that lipid peroxidation is a response to guanidino intoxication (Fig. 1,*i*). Butylated hydroxytoluene is also reported to have anti-lipid peroxidase properties (Shih and Hu, 1999).

The significant reduction of taurine's effect on GSA-dependent swelling by GES at concentrations up to 10 mmol/l (Fig. 2, *a*,*b*) indicates that taurine transport is necessary for it to be fully ameliorative. In this respect the neurotoxic mechanisms of uraemic encephalopathy appear to differ from those of hepatic encephalopathy (Zielińska *et al.,* 2003). But the partial nature of this response suggests the existence of a second, transport-independent and presumably extracellular, mode of action for taurine. This may involve the activation of $GABA_A$ receptors, which has previously been demonstrated by ligand-binding and electrophysiological techniques (Quinn and Harris, 1995; Del Olmo *et al.,* 2000). On the basis of a previously proposed model (De Deyn *et al.,* 2001, 2003) taurine may be envisaged as activating these receptors, with consequent cellular hyperpolarization. One effect of this would be retention of the voltage-sensitive Mg^{2+} block on guanidino-sensitive NMDA receptor channels, thus preventing massive cellular Ca^{2+} influx and cell damaging NOS activation. It should be noted that the NOS inhibitor NNA completely blocked guanidino-activated selling (Fig. 1, *j*). [The failure of ADMA, a probable uraemic toxin, to cause swelling (Table 1) may be due to it's NOS-inhibitory property (De Deyn *et al.,* 2003). Alternatively, the concentration used in this study may have been inappropriately low.] The partial abolition of taurine's protective effect by the $GABA_A$ receptor antagonist bicuculline (Fig. 2, *d)* is consistent with the involvement of these receptors. The fact that GES and bicuculline appear to act additively in completely abolished the ameliorative effect of taurine (Fig. 2, *e*) is consistent with the existence of both intra- and extracellular protective mechanisms.

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PROPERTIES OF BASAL TAURINE RELEASE IN THE RAT STRIATUM *IN VIVO*

Svetlana M. Molchanova, Simo S. Oja, and Pirjo Saransaari*

1. INTRODUCTION

Taurine (2-aminoethanesulphonic acid) is a non-essential amino acid abundant in the central nervous system of mammals. Due to its numerous neuronal effects, taurine is starting to engross researchers' attention as the possible inhibitory neurotransmitter. Application of taurine generally modifies neuronal electrical potentials by increasing Cl^- conductance and evokes hyperpolarization (Oja *et al.,* 1990; Belluzzi *et al.,* 2004). Exogeneously applied taurine regulates the intracellular calcium increase induced by glutamate and affects the activity of calcium-dependent enzymes (Foos and Wu, 2002; El Idrissi and Trenkner, 2004; Wu *et al.,* 2005). Although taurine may exert the influence on GABA and glycine receptors (Malminen and Kontro, 1986; Kontro and Oja, 1987c; Ye *et al.,* 1997; Belluzzi *et al.,* 2004), the existence of specific taurine receptor, which is probably the agonist-activated chloride channel, is widely discussed (Kontro and Oja, 1983, 1987a, 1987b; Frosini *et al.,* 2003a, 2003b).

Many specific questions, however, remain open, hampering clear conclusions as to the neurotransmitter or neuromodulatory functions of taurine. One of the most important questions is the regulation of the extracellular taurine concentration *in vivo*. In case of classical neurotransmission, the substance should be released from neurons upon depolarization through Ca^{2+} dependent exocytosis and then rapidly removed form the extracellular space. The present data on the origin and mechanism of the evoked taurine release are inconsistent. Many studies have failed to witness the neuronal origin of the K^+- or NMDA-evoked taurine release (Hanretta and Lombardini, 1987; Semba *et al.,* 1995). Taurine release is invariably slower in onset and offset (Korpi *et al.,* 1981; Kontro and Oja, 1987d). The data concerning the accumulation of taurine in synaptic vesicles are somewhat contradictory (Kontro *et al.,* 1980; Fyske and Fonnum, 1996). However, to

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fulfill neuromodulatory functions in the brain, the extracellular taurine pool should be well compartmentalized and strictly regulated by neuronal activity, regardless of the mechanisms. This paper focuses on the characteristics of extracellular taurine pool and the mechanisms providing its dynamic regulation.

2. EXTRACELLULAR TAURINE POOL

Taurine abounds in the brain of many mammalian species (Oja and Kontro, 1983; Huxtable, 1989). Its tissue concentration was shown to be between 5-60 nmoles per mg of protein (Kreisman and Olson, 2003; Lima *et al.,* 2004). Using the zero-net-flux approach, we have shown that under resting conditions the extracellular taurine concentration in the striatum amounts to 25.2 ± 5.1 μM (Molchanova *et al.,* 2004a). Our results correspond well to the earlier data obtained for the rabbit olfactory bulb and rat dentate gyrus (Jacobson and Hamberger, 1985, Lerma *et al.,* 1986). The physiological level of taurine in the extracellular space in these brain areas was shown to be between 8 and 20 μM.

The obtained values for extracellular taurine concentrations seem to be quite high compared to the concentrations of other neurotransmitter amino acids. Extracellular glutamate concentration in the striatum was shown to be around 1-3 μM (Miele *et al.,* 1996; Lai *et al.,* 2000), which is more than five times less than estimated extracellular concentration of taurine in the same brain region. Extracellular concentrations of putative amino acid neurotransmitters in the dentate gyrus are also in the low micromolar range (0.8-2.9 μM, Lerma *et al.,* 1986). However, interstitial concentrations of the non-neuroactive amino acids are much higher than that of taurine, and are in the high micromolar range. The values of intra/extracellular concentration ratios formed three groups: more than 2000 (putative neurotransmitters), less than 100 (non-neurotransmitter amino acids) and about 400 (taurine). Based on the amount of extracellular pool and the ratio of intra/extracellular concentrations, taurine keeps the intermediate position between neuroactive and non-neuroactive compounds.

3. UPTAKE OF EXTRACELLULAR TAURINE

Due to the hydrophilic molecular structure, taurine poorly penetrates the lipid bilayer of the plasma membrane. Because of the impossibility of physical diffusion, the extracellular taurine pool is likely regulated by the specific mechanisms, which include the release and uptake of taurine by neuronal cells. The uptake of taurine is performed by the active sodium-mediated transport systems. The taurine transporter (TauT), mediating taurine influx into cells, is nowadays well characterized. As well as the glutamate transporter, it belongs to the family of sodium- and chloride-dependent neurotransmitter transporters (Tappaz, 2004). The uptake is driven by the membrane gradient of Na^+ and Cl^- and regulated by the Ca^{2+}-calmodulin, protein kinase C and cAMP/protein kinase A pathways.

Using the taurine transport inhibitor guanidinoethanesulfonate (GES) we demonstrated the active uptake of taurine under resting conditions *in vivo* (Molchanova *et al.,* 2004b). Application of 1 mM GES significantly elevates the basal extracellular taurine concentrations (Fig. 1). The presence of active taurine uptake in the resting

conditions *in vivo* is similar to the uptake of other neurotransmitter amino acids. For example, the basal concentration of glutamate is elevated by the inhibition of transport systems (Rawls and McGinty, 1997, Janáky *et al.,* 2001).

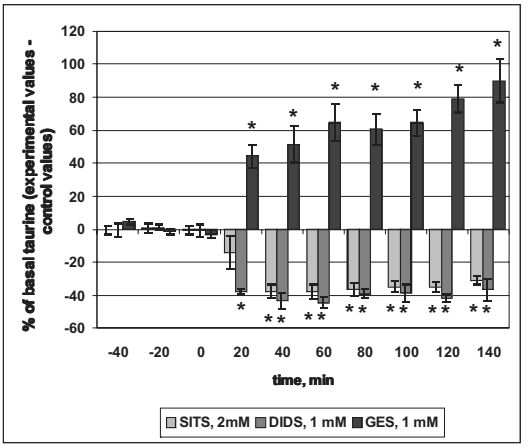

Figure 1. Regulation of extracellular taurine pool by volume-sensitive chloride channels and taurine transporter. Extracellular taurine was measured by *in vivo* microdialysis in the striatum of rats. 4-Acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid (SITS), diisothiocyanostilbene-2,2'-diulfonate (DIDS) and guanidinoethanesulfonate (GES) were perfused through the microdialysis probe after collection of three basal samples. Data are presented as mean values ± S.E.M. in percent of the basal taurine concentrations. * $p < 0.05$ compared to the control values.

There is a significant body of evidences that TauT may mediate not only uptake, but also release of taurine. All stimuli, affecting the membrane ion gradient, decrease or reverse the activity of the transporter enhancing taurine efflux. Na^+-free medium has enhanced taurine release via the reverse functioning of TauT in different preparations (Oja *et al.,* 1985; Oja and Kontro, 1987; Takuma *et al.,* 1996), including the release under ischemic conditions (Saransaari and Oja, 1998, 1999a; Phillis and O'Regan, 2003). The N-methyl-D-aspartate-evoked taurine release is mediated by the NO/cGMP pathway and the reverse action of TauT (Saransaari and Oja, 1999b; Oja and Saransaari, 2000; Scheller *et al.,* 2000). We have proved the participation of TauT in Ca^{2+} depletion-evoked taurine release (Molchanova *et al.,* 2005).

4. RELEASE OF TAURINE

The release of taurine from neural tissue could be mediated by several mechanisms, including exocytosis of vesicular taurine from nerve endings, leaking through membrane channels, and carrier-mediated transport (Oja and Saransaari, 2000). Under resting conditions the taurine carrier is active and performs the uptake of taurine inside the cell. In what follows, we consider the involvement of the exocytotic mechanisms and leaking through the membrane channels in the basal taurine release.

4.1. Membrane Channel-Mediated Release

Taurine release evoked by ischemia (Phillis *et al.,* 1997; Saransaari and Oja, 1998) and hypo-osmotic stimuli (Pasantes-Morales *et al.,* 1990; Estevez *et al.,* 1999) is partially mediated by volume-sensitive chloride channels (VSChCs). Anion channel blockers 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid (SITS) and diisothiocyano-stilbene-2,2'-diulfonate (DIDS) are the blockers of VSChCs. Both compounds decrease the extracellular taurine concentration *in vivo* (Fig. 1; Molchanova *et al.,* 2004b, 2005). We infer from the obtained data that the extracellular taurine pool is supplied by the exit of taurine through volume-sensitive anion channels and about one half of taurine exits through these channels under resting conditions.

4.2. Exocytotic Release

The data on the synaptic release of taurine are still contradictory. The synaptosomal fraction is highly enriched by taurine (Kontro *et al.,* 1980). However, the active uptake of taurine into the synaptic vesicles is not shown (Fyske and Fonnum, 1996). Taurine is released from nervous tissue in response to depolarizing stimuli, e.g., increased extracellular K^+ concentrations, veratridine and agonists of glutamate receptors (Kontro and Oja, 1987d; Saransansaari and Oja, 1997, 1999c, 2003; Molchanova *et al.,* 2004a). This depolarization-evoked release is apparently consistent with the exocytotic liberation of taurine from neural preparations. To ascertain the involvement of synaptic mechanisms in the basal release of taurine, we have estimated the effect of inhibition of voltage-sensitive sodium channels by tetrodotoxin (TTX) and the reduction of the Ca^{2+} uptake by the Ca^{2+}-free medium on the basal taurine release (Molchanova *et al.,* 2004b). The release of glutamate was used in these experiments as a positive reference.

We have shown that the extracellular concentrations of both glutamate and taurine were increased by TTX application (Molchanova *et al.,* 2004b). Previous results on TTX effects on taurine and glutamate release are discrepant. For instance, the basal release of taurine in the substantia nigra *in vivo* and from brain slices *in vitro* has been slightly reduced or unaffected by inhibition of voltage-gated Na^+ channels by TTX (Biggs *et al.,* 1995; Bianchi *et al.,* 1999). The kainate-stimulated taurine release was insensitive to the inhibition of voltage-regulated sodium channels (Bianchi *et al.,* 1998). In several studies no changes or a minor decrease in the glutamate concentration have been detected in response to inhibition of the voltage-gated sodium channels (for review see Timmerman and Westerink, 1997). However, in those studies specifically conducted on the striatum *in vivo*, extracellular glutamate was increased under TTX application (Keefe *et al.,* 1993; Morari *et al.,* 1996). This phenomenon may reflect the TTX-mediated disinhibition of cortical glutamatergic terminals. The firing of cortical and thalamic motoneurons is controlled by the striato-thalamo-cortical loop (Parent and Hazrati, 1995a, 1995b). Under resting conditions the striatal efferents are silent, leaving thalamic neurons suppressed. The dopaminergic striatonigral projections, which are tonically active, balance the activity of the two branches of the loop. Intrastriatal application of TTX attenuates dopaminergic tone and leads to disinhibition and subsequent release of glutamate from the cortical inputs.

In our studies taurine was released upon TTX application in a similar manner and in the same order of magnitude as glutamate (Fig. 2). Taurine is thus either released from the same site (cortical inputs) or glutamate induces taurine release from other sources. It

has previously been reported that decortication reduces the striatal levels of glutamate, but leaves the taurine concentrations unchanged (Butcher and Hamberger, 1987). The TTX-induced taurine elevation is hence likely to occur secondarily after the glutamate release. Although taurine may be released by a non-synaptic mechanism, the release is strongly coupled to changes in neuronal activity.

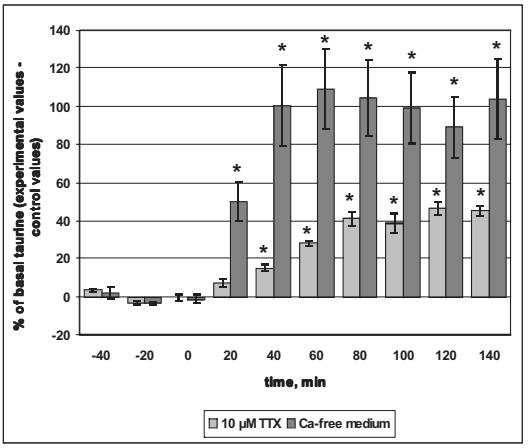

Figure 2. Regulation of the extracellular taurine pool by changes in neuronal activity. Extracellular taurine was measured by *in vivo* microdialysis in the striatum of rats. Tetrodotoxin (TTX) and Ca^{2+}-free medium were perfused through the microdialysis probe after collection of three basal samples. Data are presented as mean values ±S.E.M. in percent of the basal taurine concentrations. * $p < 0.05$ compared to the control values.

Depolarization-evoked release has been shown to be partially Ca^{2+}-dependent, suggesting the involvement of the exocytotic pathway (Kamisaki *et al.,* 1993; Menéndez *et al.,* 1993). However, a number of other investigators have failed to show the Ca^{2+} dependency of evoked taurine release (Holopainen *et al.,* 1985; Solís *et al.,* 1986). The main factor hampering clear conclusions on Ca^{2+} dependency is that Ca^{2+} depletion significantly enhanced the basal release of taurine in those studies. Nevertheless, when K^+-stimulated release under Ca^{2+}-free conditions is compared to the basal release (Ca^{2+}-free conditions only), a significant reduction in the evoked release in the absence of Ca^{2+} is evident (Korpi and Oja, 1984; Oja *et al.,* 1985; Oja and Kontro, 1987).

The basal taurine release, measured in the absence of any stimuli, is enhanced in extracellular Ca^{2+}-free medium (Fig. 2). Using superfused brain slices and astrocytes in culture, it has been shown *in vitro* that Ca^{2+}-free medium elevates extracellular taurine and that Ca^{2+} chelators enhance this effect (Korpi and Oja, 1984; Oja *et al.,* 1985; Saransaari and Oja 1992; Takuma *et al.,* 1996). We confirmed these data using *in vivo* microdialysis (Molchanova *et al.,* 2004b, 2005). The observed effect is specific for taurine; omission of Ca^{2+} reduced the extracellular glutamate concentrations.

Due to methodological problems it is difficult to prove the Ca^{2+} dependency of the release measured by microdialysis. The basal glutamate release has been found to be unchanged or only slightly decreased after omission of Ca^{2+} (for review see Timmerman and Westerink, 1997). We have shown a minor but statistically significant decrease in

extracellular glutamate, which was likewise discernible in Ca^{2+}-free medium. In contrast, taurine release was significantly enhanced in the absence of Ca^{2+}. Interestingly, the omission of Ca^{2+} has no effect on taurine release in the supraoptic nucleus of the hypothalamus, where taurine is localized mainly in glia (Decavel and Hatton, 1995; Hussy *et al.,* 2000). Since the localization of taurine in the striatum is predominantly neuronal (Storm-Mathisen and Ottersen, 1986; Madsen *et al.,* 1987; Della Corte *et al.,* 1990), enhanced taurine release in Ca^{2+}-free solution may be a phenomenon specific for neurons, but not for glia.

Summarizing, inhibition of synaptic transmission by the voltage-sensitive Na^{+} channel blocker tetrodotoxin and Ca^{2+} depletion results in an increase in extracellular taurine, suggesting that synaptic exocytosis is not involved in the regulation of the extracellular pool of this amino acid under basal conditions. These results indicate taurine release to be related to neuronal activity, although in a non-classical way. However, the obtained results do not exclude the involvement of synaptic mechanisms in the stimulated taurine release.

5. BASAL TAURINE RELEASE UNDER CALCIUM-FREE CONDITIONS

The effect of Ca^{2+} on the extracellular taurine concentrations is of special interest in the context of the regulatory mechanisms of taurine release. In the further studies we characterized the basal release of taurine in Ca^{2+}-free medium in the rat striatum *in vivo* and determined the driving force underlying the enhanced release of taurine under these conditions (Molchanova *et al.,* 2005).

Taurine can be released by several pathways, two of them being the most prominent under normal conditions: the release through volume-sensitive chloride channels and the reverse action of TauT. Both of these types may be responsible for the elevated efflux of taurine under Ca^{2+} depletion. Under resting conditions at least one half of extracellular taurine exits cells through VSChCs. This exit is further enhanced under hypo-osmotic conditions (Estevez *et al.,* 1999). However, the upregulation of VSChCs under these conditions has been shown to be essentially Ca^{2+}-independent (Pasantes-Morales and Morales Mulia, 2000). Takuma and associates (1996) have demonstrated that Ca^{2+} depletion releases taurine through volume-independent mechanisms, since Ca^{2+}-free medium does not cause any cell swelling. Furthermore, the K^{+}/Cl^{-} co-transport inhibitor furosemide and sucrose did not affect taurine release under Ca^{2+} depletion. In our study, in the presence of the VSChC blocker DIDS, the omission of Ca^{2+} evoked the release of taurine of the same magnitude when compared to the release in Ca^{2+}-free artificial cerebrospinal fluid (aCSF) alone (Fig. 3). The data obtained confirm the conclusion of Takuma and associates (1996) that osmoregulatory mechanisms are not involved in the studied phenomenon.

Since Ca^{2+} depletion changes the membrane gradient of Na^{+} ions, the efflux of neurotransmitters in the absence of calcium can be mediated by transport systems operating in a reverse mode (Bernath, 1992). Takuma and colleagues (1996) were the first to propose that TauT provides for the exit of taurine from cells in Ca^{2+}-free medium. To test whether the TauT pathway of taurine release is involved under Ca^{2+}-free conditions, we inhibited its reverse action by GES loaded into the cells. After a one-hour application of GES, Ca^{2+}-free medium did not evoke any significant elevation in the

interstitial taurine concentration (Fig. 3). These data confirm that Ca^{2+} depletion reverses TauT and enhances taurine release from the cells.

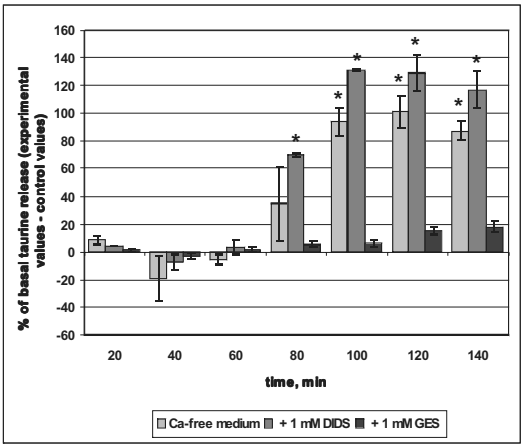

Figure 3. Mode of taurine release under Ca^{2+} depletion. Extracellular taurine was measured by *in vivo* microdialysis in the striatum of rats. Diisothiocyanostilbene-2,2'-disulfonate (DIDS) was perfused through the microdialysis probe during the whole experiment and guanidinoethanesulfonate (GES) during the first hour. Ca^{2+}-free medium was perfused after collection of three basal samples. Data are presented as mean values ± S.E.M. in percent of the basal taurine concentrations. * $p < 0.05$ compared to the control values, containing the corresponding compounds in the normal aCSF.

The Ca^{2+} dependency of neurotransmitter release is based on the inhibition of Ca^{2+} entry via voltage-sensitive calcium channels (VSCCs) and subsequent decrease in $[Ca^{2+}]_i$ (Berridge *et al.,* 2003). We have tested whether the inhibition of Ca^{2+} entrance through VSCCs underlies the enhancement of taurine release in the absence of Ca^{2+}. Both Cd^{2+} and Ni^{2+} at micromolar concentrations are potent inhibitors of VSCCs (Yamakage and Namiki, 2002). Cd^{2+} has the higher affinity for the L/N types of VSCCs, while Ni^{2+} is a more potent inhibitor of the P/Q channels. Blocking of the VSCCs of P/Q type in the presence of extracellular Ca^{2+} elevates the extracellular taurine concentration (Fig. 4). Since the direct VSCC inhibition mimics the effect of Ca^{2+} omission from the extracellular space, we conclude that a part of the taurine release evoked by Ca^{2+}-free medium is due to a decrease in Ca^{2+} flow through the VSCCs of P/Q type.

We have shown that the inhibition of VSCCs evokes taurine release. One could thus expect VSCC blockers to enhance the effect of Ca^{2+}-free medium. On the contrary, both $CdCl_2$ and $NiCl_2$ attenuate the effect of Ca^{2+} omission (Fig. 4). These data are in accordance with previous findings. In particular, the VSCC blocker nifedipine has been shown to attenuate the Ca^{2+} depletion-evoked release of taurine from astrocytes (Takuma *et al.,* 1996). The selectivity of VSCCs for Ca^{2+} is particularly high. It binds to the pore walls and the influx of this ion through VSCCs is highly specific (Sather and McCleskey, 2003). The presence of Ca^{2+} inside the pore eliminates nonspecific flow through VSCCs.

However, other ions of smaller size are able to penetrate VSCCs in absence of Ca^{2+}. Under these conditions a fast but non-specific flow of Na^+ through VSCCs has been

described (Almers *et al.,* 1984; Fukushima and Hagiwara, 1985). We suggest that under calcium depletion this nonspecific efflux of Na^+ disrupts the membrane gradient of sodium ions, reverses TauT and thus initiates the release of taurine.

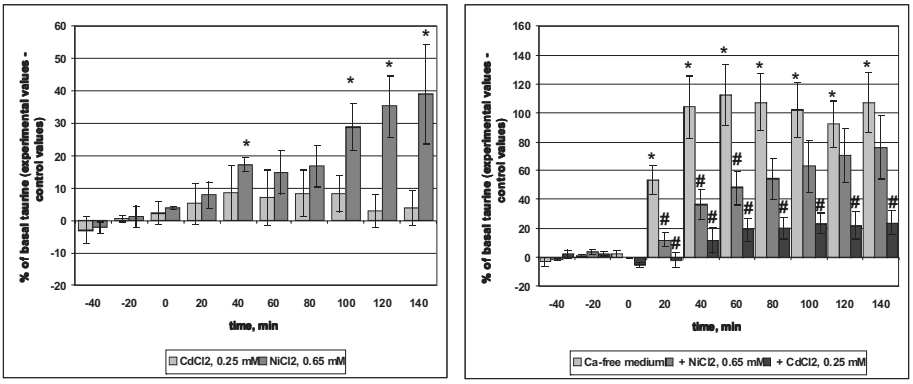

Figure 4. Effects of Ca^{2+}-free medium and voltage-sensitive calcium channel blockers on the release of taurine. Extracellular taurine was measured by *in vivo* microdialysis in the striatum of rats. Ca^{2+}-free medium and metal salts were perfused through the microdialysis probe after collection of three basal samples. Data are presented as mean values ± S.E.M. in percent of the basal taurine concentrations. * $p < 0.05$ compared to the control values; # $p < 0.05$ compared to the values under Ca^{2+} depletion.

6. CONCLUSIONS

The extracellular pool of taurine is large in comparison with the putative neurotransmitters. Under resting conditions it is dynamically regulated by the uptake and release of taurine by neuronal cells. The uptake is carried out by the taurine transporter, which is actively working under resting conditions. However, the uptake process can be reversed by even small changes in the membrane ion gradient, e.g. evoked by the omission of Ca^{2+} ions. Volume-sensitive chloride channels mediate the release of taurine under resting conditions.

Synaptic exocytosis is not involved in the release of taurine under resting conditions. The inhibition of neuronal activity does not reduce, but elevates the extracellular taurine in vivo. The amount of extracellular taurine pool particularly depends on the extracellular concentration of Ca^{2+}. Taurine release is enhanced by the reduced influx of Ca^{2+} through the taurine transporter operating in the outward direction. A major part of taurine release is driven by the nonspecific influx of Na^+ through voltage-sensitive Ca^{2+} channels. This nonspecific influx is likely an artifact generated by the experimental setup, since the absence of Ca^{2+} never occurs under physiological conditions. A minor part of release is evoked by the decreased influx of Ca^{2+} through voltage-sensitive Ca^{2+} channels. These data indicate a particular role of taurine transporter in the release of taurine evoked by neuronal activity.

To summarize, it was proved that the mechanism of the regulation of extracellular taurine pool is not the same as that of classical neurotransmitters. However, extracellular taurine is affected by changes in the neuronal activity, allowing taurine to fulfill the neuromodulatory functions.

7. ACKNOWLEDGMENTS

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NEUROPROTECTIVE MECHANISMS OF TAURINE *IN VIVO*

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1. INTRODUCTION

The physiological actions of taurine have received attention since the original experiments of Curtis and Watkins (1960, 1961) on the synaptic effects of excitatory and inhibitory amino acids. The significance of taurine in brain functions was thereafter sporadically emphasized (e.g., Oja and Piha, 1966; Davison and Kaczmarek, 1971, Lähdesmäki and Oja, 1972; Oja *et al*., 1977). It is also apparently involved in many other physiological functions (Oja and Kontro, 1983; Huxtable 1992; Oja and Saransaari, 1996). Taurine abounds in the developing brain, serving as a trophic factor (Sturman *et al*., 1986; Palackal *et al*., 1991). It induces hyperpolarization and inhibits firing of central neurons and has been thought to act as a modulator of synaptic activity in the brain (Oja *et al*., 1977; Kuriyama, 1980; Oja and Kontro, 1983; Saransaari and Oja, 1992). Taurine also acts as an osmoregulator (Solis *et al*., 1988, Wade *et al*., 1988) and possesses an antioxidant role, attenuating oxidative damage to DNA (Messina and Dawson, 2000) and protecting against menadion-induced oxidative stress (Devamanoharan *et al*., 1998). The maintenance of the integrity of membranes (Pasantes-Morales and Cruz, 1985; Moran *et al*., 1988), regulation of Ca^{2+} binding and transport (Lazarewicz et al., 1985; Lombardini, 1985) and transmembrane Cl^- flux (Taber *et al*., 1986) are also probable functional features of taurine in the brain.

Taurine has beneficial effects under a variety of cell-damaging conditions, but the protection mechanisms are still unknown. Under normal conditions taurine is generally tightly retained inside brain cells but its release is markedly increased in ischemic injury, hypoglycemia and hypoxia and upon exposure to free radicals and oxidative stress (Saransaari and Oja, 2000). This release under critical conditions indicates that the cell attempts to protect itself. Our present study focused on the neuroprotective activity of taurine in *in vivo* models of acute neurotoxicity induced by ammonia (Butterworth, 1993; Albrecht 1998) and d-amphetamine (Heikkilä *et al*., 1975; Ricaurte *et al*., 1982; Albers and Sonsalla, 1995).

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2. MATERIALS AND METHODS

2.1. Animals

Adult male Sprague-Dawley rats, 200-250 g (Orion, Espoo, Finland) were given food and water *ad libitum* and maintained in a temperature-controlled room (22±1°C) with constant relative humidity (50%) under a 12-h light/dark cycle. The procedures were conducted in accordance with the guidelines set by the European Community Directive for the ethical use of experimental animals (86/609/EEC). All efforts were made to minimize both the suffering and the number of animals used.

2.2. Models of Neurotoxicity

Repeated acute toxic doses of d-amphetamine or another psychostimulant sydnocarb (four subsequent intraperitoenal injections two hours apart) were used as a model of neurotoxicity. d-Amphetamine (Sigma, St. Louis, MO, USA) was dissolved in 0.85% NaCl and sydnocarb (Russian Center for Drug Chemistry, Moscow, Russia) in 0.85% NaCl-propyleneglycol mixture (50/50, v/v) (Witkin *et al*., 1999). d-Amphetamine was given at a dose of 5 mg/kg and sydnocarb at the equimolar dose of 23.8 mg/kg.

Direct infusion of ammonium chloride through the microdialysis probes into the rat brain was used as a model of acute ammonia-induced toxicity. N-Methyl-D-aspartate (NMDA) was used as a reference drug. Sixty mM ammonium chloride or 1 mM NMDA and/or 85 mM taurine were infused for 40 min. The extracellular concentrations of ammonia and taurine during infusions were 5 mM and 10 mM, respectively, when corrected for the probe efficiency (Zielińska *et al*., 2002).

2.3. Microdialysis

The rats were anesthetized with 4% halothane in air within 2 min and then maintained under anesthesia with 1% halothane in air delivered at 1.2 l/min. Microdialysis probes of concentric design (0.5 mm o.d., 2-mm dialysing membrane, CMA 12, CMA/Microdialysis AB, Stockholm, Sweden) were implanted into the left and right caudate-putamen (coordinates from bregma, AP= +0.5, ML= ±0.3, DV= -6.5 according to the atlas of Paxinos and Watson (1986). They were perfused at 2 µl/min with artificial cerebrospinal fluid (CSF) (composition in mM: Na^+ 150; K^+ 3.0; Ca^{2+} 1.4, Mg^{2+} 0.8; PO_4^{3-} 31.0; Cl^- 155; pH 7.4) for 1 to 2 h before commencement of sample collection, maintaining a constant flow rate with a microdialysis pump (CMA/Microdialysis) throughout the experiments. Dialysate fractions were collected at every 20 min. The location of the microdialysis probes in the brain was verified *post mortem* with cryostate microtome sections.

2.4. Analyses

The amino acids were assayed from dialysates kept frozen at -70°C and thawed immediately prior to the analyses. The concentrations of glutamate, glycine and taurine were measured by high-performance liquid chromatography with fluorescence detection (Shimadzu Scientific Instruments, Kyoto, Japan) after pre-column derivatization with o-phthalaldehyde (Sigma, St. Louis, MO, USA) (Kendrick *et al*., 1996). Derivatization was

performed with an autoinjector SIL-10AD (Shimadzu Scientific Instruments, Kyoto, Japan). The samples in the autoinjector were maintained at 4°C by a Peltier thermoelectric sample cooler. The separation column was C18-HC (ODS, 5 μm packing, 4.6 mm i.d. x 25 cm, Waters, UK) equipped with a guard column (4x20 mm). The mobile phase contained 0.075 M phosphate buffer (pH 6.5); methanol and acetonitrile were used as organic eluents with the gradient profiles of 14-25 % and 0-10 %, respectively. The amino acid derivatives were assayed using an RF-10A fluorescence detector with excitation and emission wavelengths set at 340 and 450 nm, respectively. The data were analyzed by PC using VPclass5 software and quantified by comparing the peak areas to these of standards.

The monoamines were separated immediately after collection by high-performance liquid chromatography and detected electrochemically in a system designed for monoamine assays (Sharp *et al*., 1986). The separation column was PR-C18 column, 3 μm packing, 3 mm i.d. x 15 cm (ESA Inc., Chelsford, MA, USA). The mobile phase contained 0.1 M citrate-phosphate buffer (pH 3.0), 1.1 mM octanesulfonic acid, 0.1 mM ethylenediaminetetra-acetate (EDTA) and 9-13% acetonitrile. The flow rate was 0.7 ml/min. Detection was done with Coulochem II (ESA Inc.) electrodes 1 and 2 set at -175 mV and +200 mV, respectively, The data were analyzed using BMDP software (BMDP Software Inc, Los Angeles, CA, USA).

The generation of hydroxyl radicals was determined by quantifying the rate of formation of 2,3-dihydroxybenzoic acid (DHBA) (a reaction product of salicylic acid and hydroxyl radicals) in the microdialysis perfusates (Obata and Chiueh, 1992). Sodium salicylate (5 mM) was dissolved in CSF at pH 7.4 immediately before perfusion.

3. RESULTS

3.1. Effects of Taurine on the Extracellular Levels of Dopamine and Its Metabolites Evoked by Intrastriatal Ammonia or NMDA Infusion

The basal levels of dopamine, dihydroxyphenylacetic acid (DOPAC) and 2,3-DHBA in the perfusates were in these experiments 1.6 ± 0.3, 375 ± 76 and 102 ± 7 nM, respectively (n=27). These values are not corrected for the recovery in the dialysate samples. The intrastriatal infusion of taurine (85 mM) alone within 40 min produced no significant changes in the extracellular level of dopamine (Fig. 1A). The extracellular level of DOPAC had a tendency to decline gradually after the taurine injection (Fig. 1B).

The intrastriatal infusions of 1 mM NMDA (Fig. 1A) and 60 mM ammonium chloride (Fig. 2A) within 40 evoked 250- to 300-fold increases in the extracellular level of dopamine, which persisted throughout the whole period of administration. When the infusion of ammonium chloride and NMDA was stopped, the extracellular level of dopamine decreased and reached the control level within 40 min. The co-infusion of NMDA (Fig. 1A) or ammonium chloride (Fig. 2A) with taurine did not affect the magnitude of enhanced dopamine release but the level promptly declined after the sharp initial elevation.

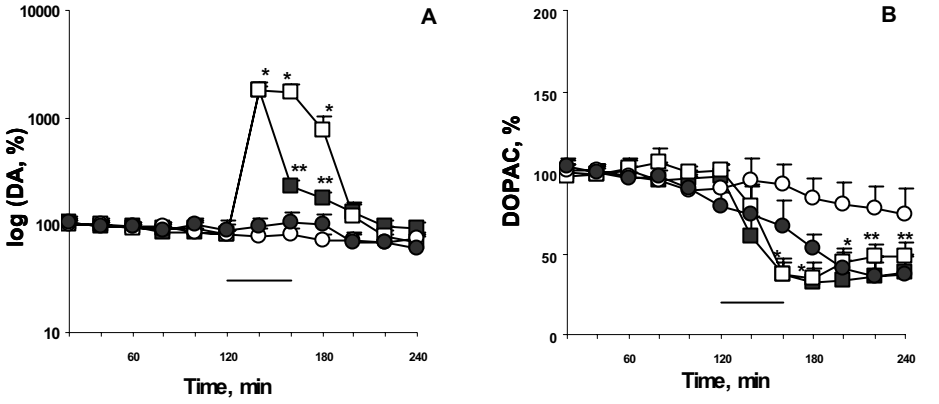

Figure 1. Effects of NMDA (1 mM, 40 min) and taurine (85 mM, 40 min) infusions on the extracellular dopamine (A) and DOPAC (B). Control (-❍-), taurine (-●-), NMDA (-□-) and NMDA plus taurine (-■-). The bars indicate the periods of NMDA and taurine infusions. Significant differences from control: *p<0.01, and between the effects of NMDA and NMDA plus taurine infusions: **p<0.05. Note the logarithmic concentration scale in panel A.

Intrastiatal infusion with NMDA (1 mM) results in a decrease in the extracellular level of DOPAC (41 ±9 %). Taurine had a tendency to prolong the effect of NMDA (Fig. 1B). In contrast, the extracellular level of DOPAC started to increase at the beginning of ammonia infusion and reached the maximal level 40 min later (217 ±29 %). The cessation of ammonia administration diminished the extracellular DOPAC level to the control value (Fig. 2B). The simultaneous administration of ammonia and taurine clearly attenuated DOPAC accumulation (138 ±16 %), but the elevation was still significant in comparison to the control.

NMDA (Fig. 3A) and ammonia (Fig. 3B) alone caused a 2.5-fold increase in the extracellular hydroxyl radical content (2,3-DHBA level) in microdialysates. A biphasic increase was observed when NMDA was injected. Taurine suppressed the first and prevented completely the second NMDA-induced increase in the hydroxyl radical levels (Fig. 3A) and taurine co-administration reduced the ammonia-induced increase to about 1.7-fold of the control (Fig. 3B).

3.2. Effects of Acute Toxic Doses of d-Amphetamine and Sydnocarb on the Extracellular Levels of Glutamate, Glycine and Taurine

The basal extracellular levels of amino acids, measured in 69 rats, were in the caudate-putamen 0.38 ± 0.14 μM, 0.62 ± 0.03 μM and 1.21 ± 0.22 μM for glutamate, glycine and taurine, respectively. These values are not corrected for the recovery in the dialysate samples. Subchronic d-amphetamine administration (5.0 mg/kg x 4, i.p.) caused a marked gradual increase in the extracellular levels of glutamate (Fig. 4, 1A) and taurine (Fig. 4, 1B) up to 400-550% and 480-580% of control, respectively. The injection of sydnocarb vehicle (propylenglycol/0.85 % NaCl) resulted in a twofold increase in the glutamate level, which was significantly different from the mean basal value (Fig. 4, 2A).

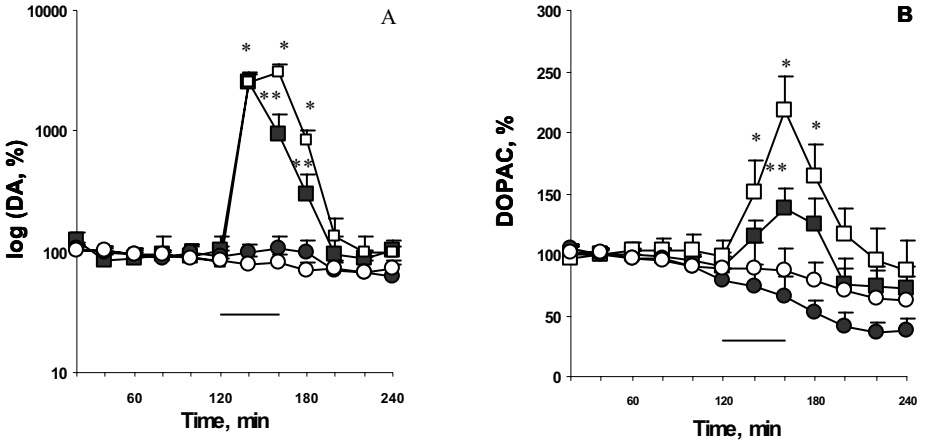

Figure 2. Effects of ammonium chloride (60 mM, 40 min) and taurine (85 mM, 40 min) infusions on the extracellular dopamine (A) and DOPAC (B). Control (-❍-), taurine (-●-), ammonium chloride (-□-) and ammonium chloride plus taurine (-■-). The bars indicate the periods of ammonium chloride and taurine infusions. Significant differences from control: *p<0.01, and between the effects of ammonium chloride and ammonium chloride plus taurine infusions: **p<0.05. Note the logarithmic concentration scale in panel A.

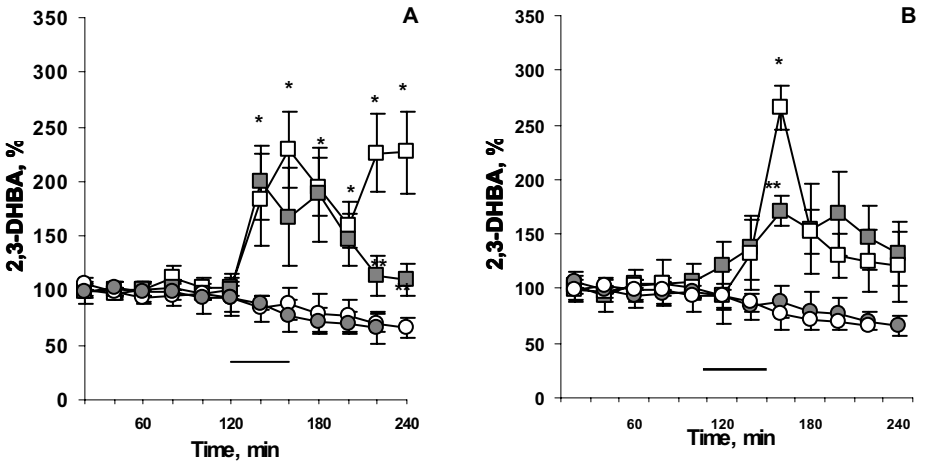

Figure 3. Effects of taurine (85 mM, 40 min) on the accumulation of 2,3-DHBA in the rat striatum evoked by NMDA (1 mM, 40 min) (A) and ammonium chloride (60 mM, 40 min) (B). Control (-❍-), taurine (-●-), ammonium chloride or NMDA (-□-) and ammonium chloride or NMDA plus taurine (-■-). The bars indicate the periods of substance infusions. Significant differences from control: *p<0.01, and between the effects of ammonium chloride or NMDA and ammonium chloride plus taurine or NMDA plus taurine infusions: **p<0.05.

Subchronic sydnocarb administration as such (23.8 mg/kg x 4, i.p.) did not result in any significant changes in extracellular glutamate. The taurine level was not altered within

the first six hours and significantly increased only after the last (fourth) injection (Fig. 4, 2B). The final extracellular level of taurine attained after sydnocarb treatment was 3 times less than that after d-amphetamine administration. The injections with d-amphetamine induced initially some increase in extracellular glycine followed by a decrease (Fig. 4, 1C). Sydnocarb elicited a moderate increase in the glycine level (Fig. 4, 2C).

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4. DISCUSSION

The mechanisms by which ammonia impairs cerebral functions are not clearly understood. A failure in brain energy metabolism and a misbalance in excitatory and inhibitory neurotransmission are thought to be involved. Ammonia affects GABA-ergic (Wysmyk *et al*., 1992; Takahashi *et al*. 1993) and dopaminergic systems (Borkowska *et al*., 1999), interacts with NMDA receptors (Borkowska *et al*., 1997; Saransaari *et al*., 1997) and triggers excitotoxic neuronal damage (Butterworth, 2002). Taurine has been shown to counteract the activation of glutamatercic receptors (Wu *et al*., 2005). Intrastriatal injections of ammonia chloride and NMDA elicited now similar increases in the dopamine level. Because taurine depresses stimulated glutamate release from neurons (Saransaari and Oja, 1994), glutamate receptors are activated less and consequently less dopamine is released in the striatum. In the present study, taurine markedly diminished the later phase of dopamine accumulation, which tallies with the delayed attenuating effect of taurine via NMDA receptors. Taurine apparently does not enhance the clearance of dopamine from the extracellular space since it does not affect striatal dopamine uptake (Kontro, 1987).

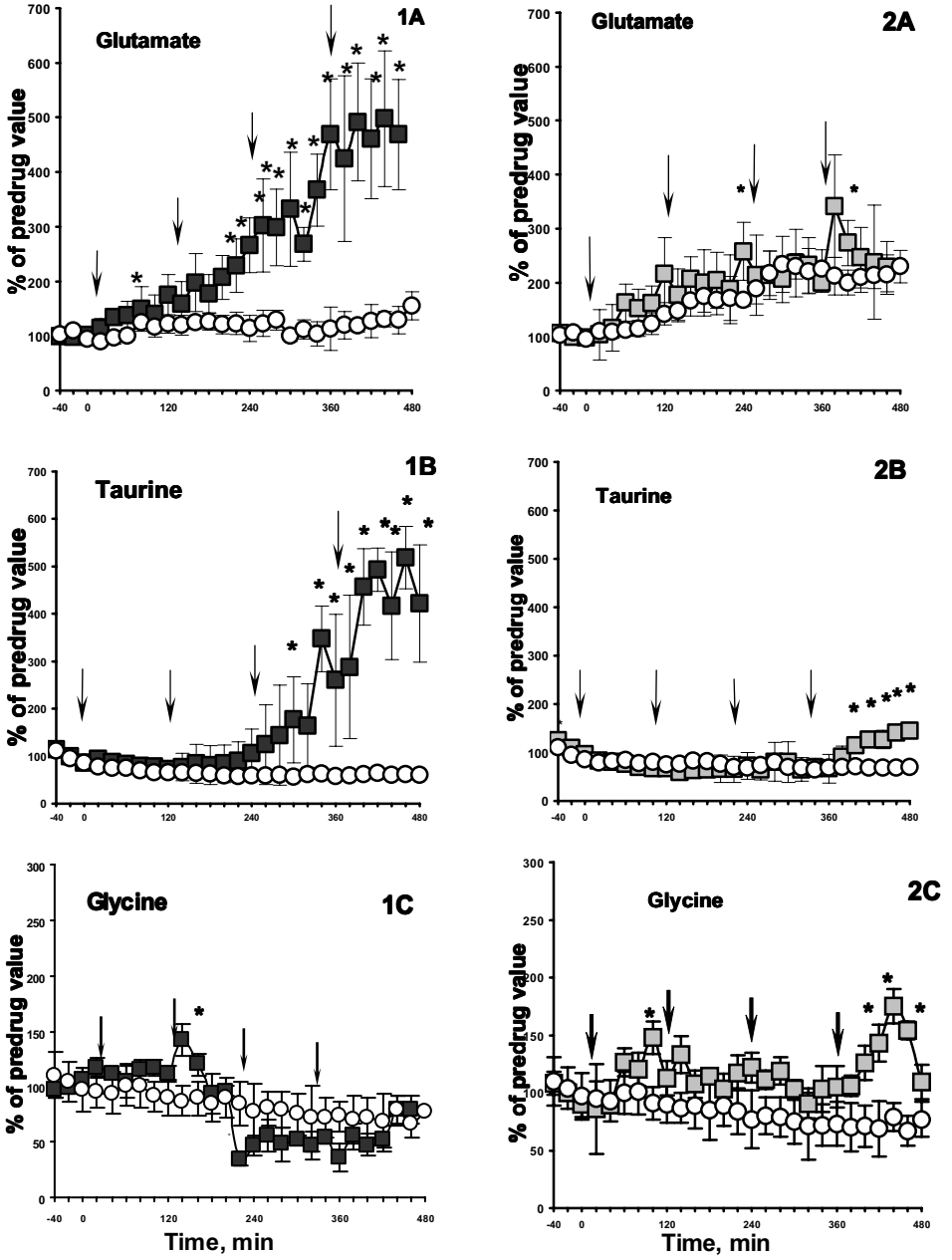

Figure 4. Effects of subchronic administration of d-amphetamine (1) and sydnocarb (2) on the extracellular levels of glutamate (A), taurine (B), and glycine (C) in the caudate-putamen in halothane-anesthetized rats Panels 1A-C: -○- 0.85% NaCl x 4, i.p.; -■- d-amphetamine, 5.0 mg/kg x 4, i.p., significant differences from control, *p<0.05, n=8. Panels 2A-C: -○- propylenglycol/0.85% NaCl x 1, i.p.; -□- sydnocarb, 23.8 mg/kg x 1, i.p., significant differences from control, *p <0.05, n=6. The arrows indicate the moments of drug administration.

In contrast to NMDA, ammonia increased the level of DOPAC, the main dopamine metabolite in the rat. A decrease in the DOPAC level is usually considered as a sign of monamineoxidase (MAO) inhibition but ammonia most probably does not exert such an effect. Presynaptic NMDA receptors are involved in the control of MAO activity. The increase in extracellular DOPAC, as a result of MAO activation after ammonia administration, must therefore originate from NMDA-independent mechanisms or only reflect the increase in extracellular dopamine.

Locally applied taurine at high concentrations elicited now an increase in extracellular dopamine and DOPAC in the striatum. Enhanced dopamine degradation is thus not probable. In contrast, taurine may interfere with the process by which ammonia activates MAO because it attenuated ammonia-induced elevation in the DOPAC level and tended to prolong the NMDA-evoked decrease. Taurine is assumed to stabilize membranes and thus may non-specifically counteract the effects of ammonia and NMDA.

Dopamine itself and its enzymatic degradation are effective sources of reactive oxygen species (Cadet and Brannock, 1998). One of the primary products of dopamine degradation, hydrogen peroxide, may generate hydroxyl radicals (via the Fenton reaction in the presence of iron and cooper) that are extremely reactive and subsequently very toxic. The decrease in the extracellular levels of dopamine and the probable inhibition of MAO may be taken into account when the effects on the levels of hydroxyl radicals are considered. On the other hand, the generation of reactive oxygen species is initiated by NMDA receptor activation, followed by NO production (Nei *et al*., 1996). NMDA and ammonia increased now the hydroxyl radical content by approximately 2.5-fold. When taurine was co-injected with NMDA or ammonia, the production of hydroxyl radicals was suppressed but not totally prevented.

The effect of taurine includes at least two components. First, taurine may serve as an antioxidant due to its radical scavenger activity. Second, taurine may prevent the interaction of NMDA or ammonia with NMDA receptors or counteract the effects of NMDA receptor activation.

Partial attenuation of the accumulation of hydroxyl radicals points to sources of reactive oxygen species in the cell milieu, in addition to NMDA receptor activation related products. The red-ox potential of taurine is relatively high to scavenge accumulated hydroxyl radicals *in vitro*. But *in vivo* the effectiveness of any antioxidant compound depends also on the compound concentration and on the presence of other antioxidants (Slater, 1979). Under some conditions taurine cannot be a final acceptor of radical products. Therefore, taurine cannot be absolutely effective in trapping reactive oxygen species. Taken together, our data may suggest that taurine is more effective as an antagonist of NMDA receptors than as a chelator of radical products in the models of ammonia- and NMDA-induced neurotoxicity.

Amphetamine liberates dopamine from cytoplasm by blocking the re-uptake. Amphetamine also pumps out dopamine from the vesicles affecting the dopamine transporter VMAT-2, which belongs to the family of toxin-extruded transporters and which alkalinises the vesicular contents (Miller et al., 1999). Ammonia ions also induce rapid alkalinization of the vesicular contents (Erecińska et al., 1987). Several subsequent injections of amphetamine in a day are enough to produce toxic damage to the brain in rodents and primates. The most interesting observation in this phenomenon of amphetamine toxicity is the selective vulnerability of dopaminergic terminals. In the clinic sydnocarb, the Russian made psychostimulatory drug, is characterized by milder stimulation, lower abuse potential and weaker sympathomimetic effects than

amphetamine. It also increases less the extracellular level of dopamine than systemically injected amphetamine at an equimolar dosage.

The strong correlation between the level of dopamine and toxic lesions has been the impetus to the dopaminergic theory of amphetamine toxicity. In the last decade glutamate has been also implicated in the pathogenesis of neuronal system dysfunctions and damage. Only subsequent injections of amphetamine, but not a single administration (Anderzhanova *et al*., 2001), result in an increase in the extracellular level of glutamate. The increase in the glutamate level correlates strongly with the increase in the extracellular concentration of taurine. The magnitude of this increase most probably reflects the toxic potential of d-amphetamine since sydnocarb elicited less elevation in the concentration of both amino acids. The increase in extracellular glutamate may be explained by the involvement of different mechanisms. First, the increased level of excitatory input in the striatum, which spreads out via projections of cortical and hypothalamic neurons may activate the basal ganglia circuitry. Second, the intrastriatal modulation of presynaptic receptors enhances glutamate release. Third, under the oxidative stress glutamate uptake is inhibited by reactive oxygen species and NO and arachidonic acid. The slowly incurring effects of these substances then underlie the delayed increase in extracellular glutamate.

Endogenous glutamate and glutamatergic receptor agonists markedly enhance taurine release (Del Arco *et al*., 2000). Upon d-amphetamine administration extracellular taurine achieved now relatively high concentrations, about 55-60 μmol, as estimated from the *in vitro* probe recovery. This level may be sufficient to counteract the effects of NMDA receptor activation *in vivo*. Striatal neurons are not particularly susceptible to the increase in extracellular glutamate or to the oxidative stress upon administration of toxic amphetamine doses. Endogenous defence, different subtypes of receptors, modulation of receptor activation and nutrients from the environment may be important. According to current knowledge, taurine may serve all these functions. Taurine is localized primarily in neurons in the striatum and may be less effective in damage prevention when present intracellularly. The d-amphetamine-induced redistribution of dopamine is critical, because it increases oxidative stress inside dopaminergic neurons. The anti-oxidative potential of taurine is not sufficient to prevent intracellular damage to neurons and although an increase in extracellular taurine seems to be crucial for its beneficial activity, the effects of released taurine on glutamatergic receptor activation cannot compensate or balance the consequences of intraneuronal lesions.

In contrast to ischemia-induced release of neuroactive amino acids (Molchanova *et al*., 2004), amphetamine tends to decrease the level of glycine in the extracellular space. Interestingly, a rapid decline in the glycine level was observed when the taurine content became about two times higher than in controls. This effect was observed after both amphetamine and sydnocarb treatments. Taurine exhibits structural similarity to glycine and GABA. Hypothetically, taurine may activate putative presynaptic glycine receptors in cholinergic interneurons, attenuating in this manner glycine release.

Taken together we may conclude the following. Sydnocarb is less toxic and elicits less glutamate release than d-amphetamine, and does not provoke marked accumulation of hydroxyl radicals. Taurine release *in vivo* is affected by endogenous glutamate. There obtains a strong correlation between the extracellular levels of glutamate and taurine. The release of taurine is a marker of toxicity and taurine itself may serve as a neuroprotective compound.

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TAURINE PARTICIPATES IN THE ANTICONVULSIVE EFFECT OF ELECTROACUPUNCTURE

Ru Yang, Qing Li, Jing-Chun Guo, Hong-Bing Jin, Jun Liu, Bu-Er Wang, and Jie-Shi Cheng*

1. INTRODUCTION

The imbalance between GABAergic inhibitory and glutamatergic excitatory amino acid systems contributes to epileptogenesis. Studies on epilepsies have been dominated by the axiom that failure of GABA-mediated inhibition generates epileptic seizure because blocking GABAergic neurotransmission induces seizures and boosting GABA controls epilepsy. However, many seizures could not be inhibited by current available antiepileptic drugs, which were designed to augment GABA transmission. It implicates that there might exist another pathway besides the potent inhibitory neurotransmitter GABA underlying epileptic bursting.

As an inhibitory amino acid, taurine has long been known to ameliorate symptoms in epilepsy (Oja and Kontro, 1983; Anyanwu and Harding, 1993) and was recently reported to suppressed epileptic activity both in animal models and tissue culture. *In vivo*, acute injection of taurine (43 mg/kg, s.c.) increased the onset latency and reduced the occurrence of tonic seizures following kainic-acid-induced epilepsy (El Idrissi *et al.,* 2003). *In vitro*, taurine suppressed seizure-like events induced by reduced extracellular Mg^{2+} concentration in rat combined entorhinal cortex-hippocampal slices (400 μm) (Kirchner *et al.*, 2003). In addition to experimental findings, taurine has been clinically used with varying degrees of success in the treatment of epilepsy and other seizure disorders (Birdsall, 1998). In Chinese clinic, traditional medicine named An-Gong-Niu-Huang-Wan is applied commonly to treat patients with epilepsy. Taurine

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is one of the major components of this pharmacy. All these data show that taurine could serve as a potential anticonvulsant.

Acupuncture has been performed in controlling epileptic seizure in clinic for thousands of years in Asia. Although acupuncture is effective and safe treatment, its application is limited because of the underlying uncertain neurobiological mechanism. In the present study, we tested whether taurine is involved in epilepsy and whether acupuncture is anticonvulsive. We investigated the release of endogenous taurine during epilepsy before and after acupuncture treatment, changes in epileptic activity after administration of taurine with/without acupuncture, and the immunoactivity of taurine transporter, which is a main vector in plasma membrane for taurine uptake from the extracellular to intracellular space.

2. MATERIALS AND METHODS

Epilepsy was induced by microinjection of 0.7 μg kainic acid into one side of lateral ventricles (P0R/L1.5H3.8) or by microinjection of 350 units of penicillin into one side of the hippocampus (P3.0R/L2.0H3.7) in adult Wistar rats (200-220 g).

Electroacupuncture was performed on a pair of acupoints of DU 16 “Feng Fu” and DU 8 “Jin Suo” or DU 20 “Bai Hui” and DU 16 “Feng Fu”. DU-16 “Feng Fu” is located at the inferior border of the occipital protuberance on the vertical midline of the posterior of the head, DU 8 “Jin Suo” at the intermediate point between the ninth and tenth thorax, and DU 20 “Bai Hui” at the middle point of the line between two ears cross the top of the head. The acupuncture needles were given by an electrical stimulation using an electroacupuncture apparatus as detailed in Yang *et al.* (2000).

Endogenous taurine was assayed by high-pressure liquid chromatography equipped with a fluorescence detector after push-pull perfusion. Exogenous taurine (20 mg/kg, 40 mg/kg and 80 mg/kg) was given intraperitioneally 25 min after penicillin.

Seizure severity was evaluated by behavior and/or electroencephalography (EEG). Epileptic seizures were classified into five stages according to Racine (1972). EEG was recorded and analyzed by differences of frequency and amplitude. The immunoreactivity of taurine transporter level was assessed in the cortex and hippocampus using immunohistochemistry.

Data were analyzed for significance using Student’s t-test. Differences with $p<0.05$ were considered statistically significant.

3. RESULTS

Electroacupuncture could inhibit epileptic events induced by both kainic acid and penicillin. EEG and behavior were recorded in penicillin-induced epileptic animals while behavior was only double-blindly evaluated in kainic-acid-induced seizures because it s difficult to record EEG in the latter model. The rats showed flattening and desynchronization in EEG and relative calmness in behavior under normal conditions. Most of the rats started an epileptiform seizure 10 min after injection of kainic acid or 25 min after injection of penicillin and the seizure lasted for more than 1 hour. With the

development of epileptic activity, the rats presented chewing, head nodding, lateral forelimb clonus, rearing of tails, screaming and even rearing and falling. Meanwhile, the frequency and amplitude of spikes or sharp waves increased in EEG. Upon administration of acupuncture, epileptic discharge was reduced in association with an improvement of rat behavior (Fig. 1).

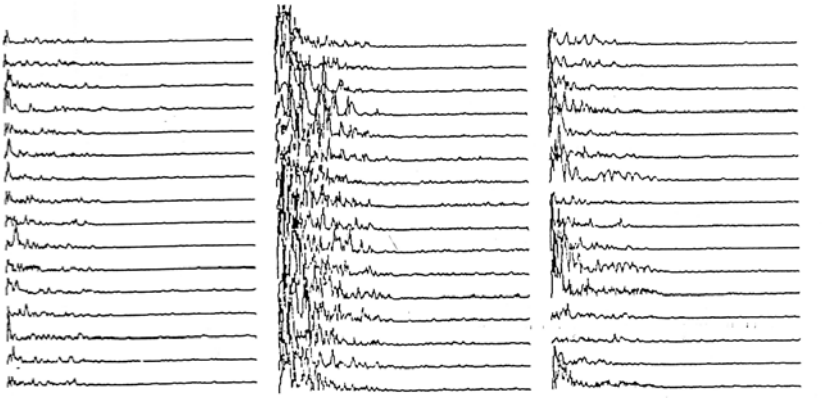


Figure 1. Electroacupuncture inhibited epileptic discharge as shown with a representative EEG power spectrum array in rats. Left pattern: normal. Middle pattern: epilepticus. Right pattern: epilepticus plus electroacupuncture.

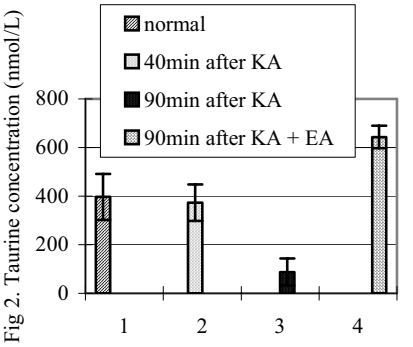


Fig 2.

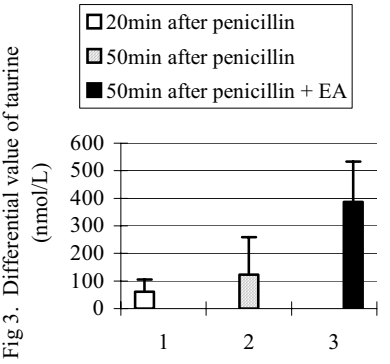


Fig 3.

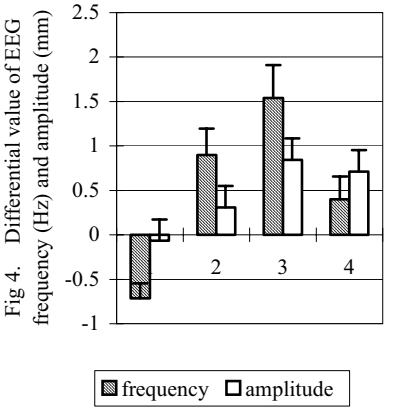
Figure 2. Electroacupuncture increased the taurine level in kainic acid-induced epilepsy ($p<0.01$). The taurine concentration tended to decrease a little but not statistically significantly at 40 min after injection of kainic acid (KA) when compared to normal controls, while it decreased significantly at 90 min after kainic acid.

Figure 3. Electroacupuncture increased the taurine level in penicillin-induced epilepsy ($p<0.05$). The taurine concentration tended to increase at 50 min after injection of penicillin when compared to rats at 20 min after penicillin. However, the possible increase was not statistically significant.

Electroacupuncture increased significantly the taurine level in the hippocampus in both kainic acid- and penicillin-induced epilepsies. In the experiments with kainic acid, the concentrations of taurine were 396.95±94.88 nmol/l (n=14), 373.16±75.19 nmol/l (n=14), 87.63±55.79 nmol/l (n=5) and 643.06±46.39 nmol/l (n=6), in normal controls, 40

min and 90 min after kainic acid administration, and 90 min after kainic acid plus electroacupuncture, respectively (Fig. 2). In the penicillin case, the differential hippocampal taurine concentrations after subtraction of the concentration under normal conditions were 61±45 nmol/l, 123±136 nmol/l and 387±146 nmol/l at 20 min and 50 min after penicillin, and 50 min after penicillin plus acupuncture, respectively (Fig. 3).

Taurine had a significant anti-epileptic effect when applied at 20 mg/kg, 40 mg/kg, and 80 mg/kg, especially at 40 mg/kg in the rat model of penicillin-induced seizure. The behavior and frequency and amplitude of EEG improved by one to three Racine grades in the animals (Fig. 4). Exogenous 40 mg/kg taurine also enhanced the anticonvulsive effect of acupuncture (Fig. 5). Both acupuncture and taurine increased the expression of taurine transporter both in the cortex and hippocampus in penicillin-induced epilepsy. Acupuncture and taurine may interact in a synergistic way (Fig. 6).



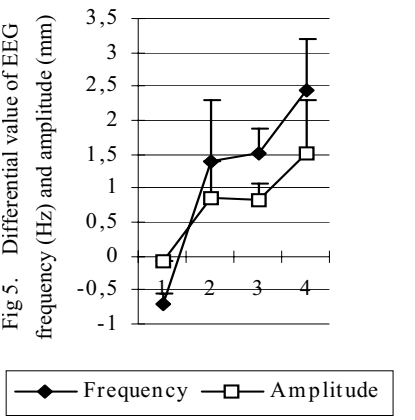


Figure 4. Exogenous taurine decreased the frequency and amplitude of EEG during penicillin treatment. The differential EEG frequency and amplitude were calculated by subtracting the frequency or amplitude at 70 min after penicillin injection from the ones at 20 min after penicillin injection. The higher the value, the lower the seizure severity. Taurine suppressed seizure discharge at 20 mg/kg (2), 40 mg/kg (3), and 80 mg/g (4) when compared to epileptic controls (1). P<0.001, penicillin *vs.* normal; 2 or 3 or 4 *vs.* 1; 3 or 4 *vs.* 2; P>0.05, 4 *vs.* 3.

Figure 5. Exogenous taurine improved the anticonvulsive effect of electroacupuncture in penicillin-induced epilepsy. The differential value of EEG frequency and amplitude were calculated by subtracting the frequency or amplitude at 70 min after penicillin injection from the ones at 20 min after penicillin injection. The higher the value, the lower the seizure severity. Both acupuncture (2) and taurine (3) inhibited the penicillin-induced seizure (1) and acupuncture plus taurine (4) inhibited synergistically the seizure. P<0.001, 2 or 3 or 4 *vs.* 1; 4. *vs*. 2 or 3; P>0.05, 2 *vs.* 3.

4. DISCUSSION

More and more evidences have emerged that taurine exhibits an inhibitory, but only slightly potent, effect on epilepsy. The dose of 40 mg/kg taurine suppressing now epileptic discharges in penicillin-induced seizures is in agreement with a recent report of the effect of 43 mg/kg in kainic acid-induced mouse limbic seizures (El Idrissi *et al.*,

2003). The neuroprotective effect of taurine has been shown by either indirect action or direct data. Taurine has prevented the neurotoxicity of glutamate receptor agonists

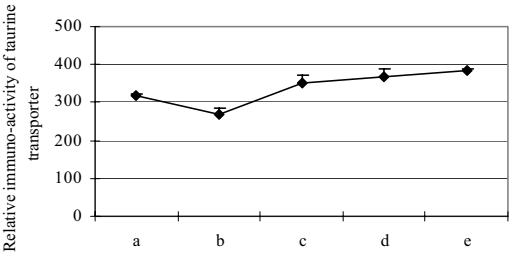


Figure 6. Electroacupuncture increased the level of taurine transporter. Immunoactive staining was measured in CA1, CA2, and CA3 areas of the hippocampus in normal controls (a), penicillin-induced epilepsy (b), acupuncture treatment after penicillin (c), taurine administration after penicillin (d), and acupuncture plus taurine after penicillin (e). $P<0.05$, c or d or e *vs.* a or b; c *vs*. e; $P>0.05$, c *vs*. d, and d *vs*. e.

(Louzada *et al.*, 2004), activated GABA receptor (Del Olmo *et al.*, 2000), and positive efficacy of taurine administration was collected in patients with epilepsy (Airaksinen *et al.*, 1980). Treatment of succinate semialdehyde dehydrogenase deficiency mice with taurine could rescue the mutant animal with generalized seizures (Hogema *et al*, 2001).

Acupuncture was documented in 'The Yellow Emperor's Classic of Internal Medicine' two thousands years ago and is still commonly practiced to inhibit epilepsy in present Chinese clinic. The synergistic antiepileptic action of acupuncture together with taurine and the release of taurine by acupuncture in this work imply that taurine may be a potential biological basis of acupuncture. Previous reports have also detected that ear-point electrical stimulation increased the contents of taurine in the hippocampus of penicillin-induced epilepsy in rats enhancing its antiepilepsy effects (Shu *et al*, 2004).

The increased taurine transporter level in the rat hippocampus and cortex by acupuncture supports the hypothesis that taurine may contribute to the anticonvulsive effect of acupuncture in epilepsy. The taurine transporter in plasma membranes, with cytosolic biosynthetic enzymes of taurine, cysteine dioxygenase and cysteine sulfinate decarboxylase, controls the cellular concentration of taurine yielding a ratio of 100-50000:1 between the inside and outside of cells (Tappaz, 2004). Acupuncture may alter the expression of the cortical and hippocampal taurine transporter increasing the intracellular taurine content. Taurine in turn may lead to alleviation of seizures through its physiological actions, including neurotransmission, neuromodulation, osmoregulation, control of calcium influx, and cell excitability. However, a tip of an iceberg has been discovered so far and we will continue our studies on the anticonvulsive effects of acupuncture and taurine.

5. ACKNOWLEDGMENT

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INVOLVEMENT OF TAURINE IN CEREBRAL ISCHEMIA AND ELECTROACUPUNCTURE ANTI-ISCHEMIA

Jingchun Guo, Peng Zhao, Yan Xia, Fei Zhou, Ru Yang, and Jieshi Cheng*

1. INTRODUCTION

Use of acupuncture for treatment of stroke has been practiced for thousands of years in China. However, basic studies on these protective mechanisms are still limited so far. The potential neurobiological pathways of acupuncture in stroke therapy remain uncertain.

During cerebral ischemia, massively released excitatory amino acids are neurotoxic and contribute to neuronal injury. Inhibitory amino acid taurine has been postulated to counteract excitotoxicity (Saransaari and Oja, 1997) and play neuroprotective roles in reducing the excessive overload of the intracellular calcium content induced by aspartate or dinitrophenol (Zhao *et al*., 1999). Taurine has also been reported to protect neurons against oxidation stress triggered by free radicals (Boldyrev *et al*., 1999) and against the neurotoxicity of β-amyloid and glutamate receptor agonists (Louzada *et al*., 2004). Our previous studies showed that electroacupuncture (EA) could ameliorate ischemic injury and further augment the interstitial taurine levels during cerebral ischemia (Zhao *et al*., 1997). The purpose of the present study was to investigate the possible relationship between taurine and the protective effect of EA against cerebral ischemia.

2. METHODS

Transient focal cerebral ischemia was induced in SD rats by middle cerebral artery occlusion (MCAO). With the modified Longa method (Longa *et al*., 1989) we inserted a 4-0 mononylon suture through the left internal carotid artery (ICA) to the origin of the

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MCA. After 2-h ischemia, the suture was withdrawn out of ICA to enable reperfusion. EA treatment was performed on DU 20 "Bai Hui" and DU 26 "Ren Zhong" immediately after the onset of ischemia and retained for 30 min and then paused for 10 min followed by additional 30 min of treatment. Taurine (80 mg/kg, i.v., Sigma) was injected 30 minutes before ischemia. To observe the effect of taurine depletion on EA anti-ischemic efficacy, the taurine analogue β-alanine was given in drinking water for 9 days at the 3% concentration. The brain taurine level was assessed by capillary electrophoresis/electrochemical detection (CE/ED). The extracellular nitric oxide (NO) level was measured with a NO-sensitive microelectrode implanted in the striatum. Bcl-2, Bax and P53 immunoreactivities were detected with immunohistochemistry. Data were analyzed for significance using Student's t-test. Differences with $p<0.05$ were considered statistically significant.

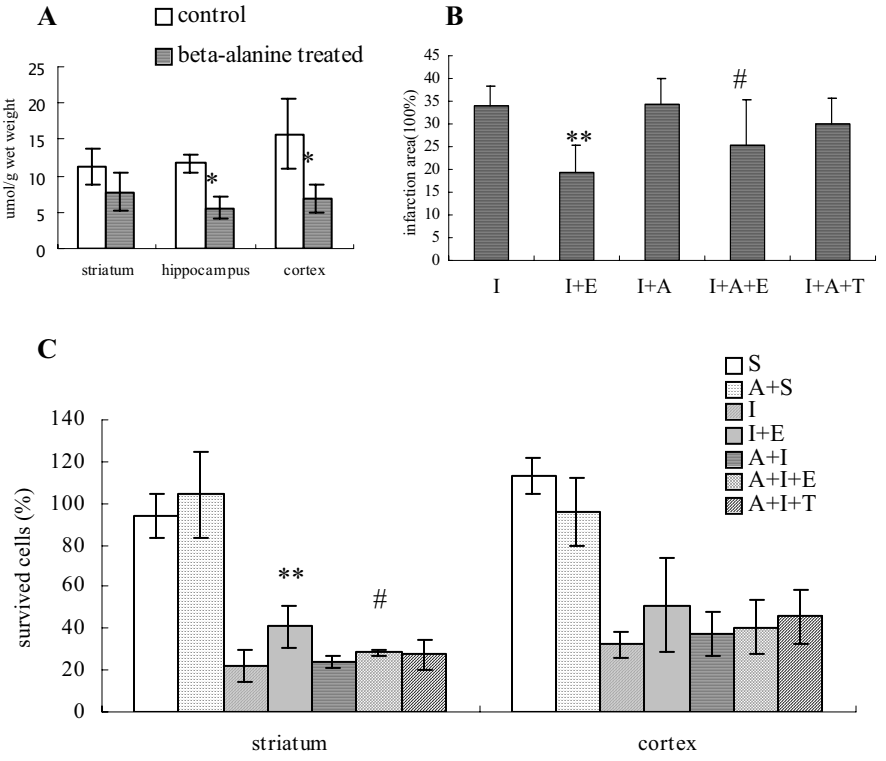

Figure 1. Effect of endogenous taurine depletion on brain infarction (B) and cell survival (C) in ischemia, and EA treatment or taurine administration. (A) Taurine levels in the cortex, hippocampus and striatum with/without 3% β-alanine treatment. S: sham-operated group; I: ischemia treated; E: EA application; A: β-alanine administration; T: taurine supplementation. *: $P<0.05$ *vs*. control group; **: $P< 0.05$ *vs*. ischemia group; #: $P<0.05$ *vs*. ischemia plus EA treatment (I+E).

3. RESULTS

After 3% β-alanine treatment the brain taurine level decreased significantly in the cortex and hippocampus (Fig. 1A). In this endogenous taurine-exhausting model taurine depletion had no effect on ischemic infarction, whereas it markedly increased the infarction in the EA-treated group (P<0.05, *vs*. EA-treated normal ischemic rats) (Fig. 1B). The effect of taurine depletion on cell survival percentage in the striatum was similar to that on infarction (Fig. 1C). We further investigated the possible protective efficacy of taurine against ischemic injury (Fig. 2). Taurine had a significant anti-ischemic effect as applied i.v. at 80 mg/kg. When compared with the effect of EA treatment, no statistical difference was found in both the infarction and cell survival percentage. However, taurine plus EA treatment exhibited a better anti-ischemic injury efficacy than either taurine or EA alone.

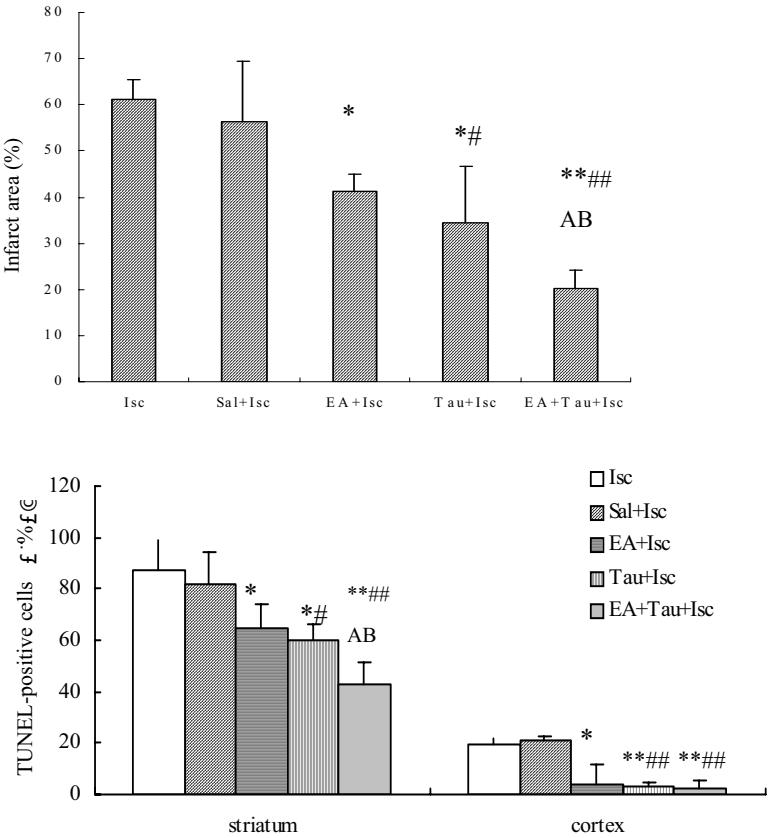

Figure 2. Effect of taurine administration on ischemic infarction (top) and percentages of TUNEL(+) cells (bottom). Isc: ischemia; EA: electroacupuncture application; Sal: saline administration; Tau: taurine administration. * P<0.05 *vs*. ischemic group; ** P<0.01 *vs*. ischemic group; # P<0.05 *vs*. saline plus ischemia group; ## P<0.01 *vs*. saline plus ischemia group; A, P<0.05 *vs*. EA plus ischemia group; B, P<0.05 *vs*. taurine plus ischemia group.

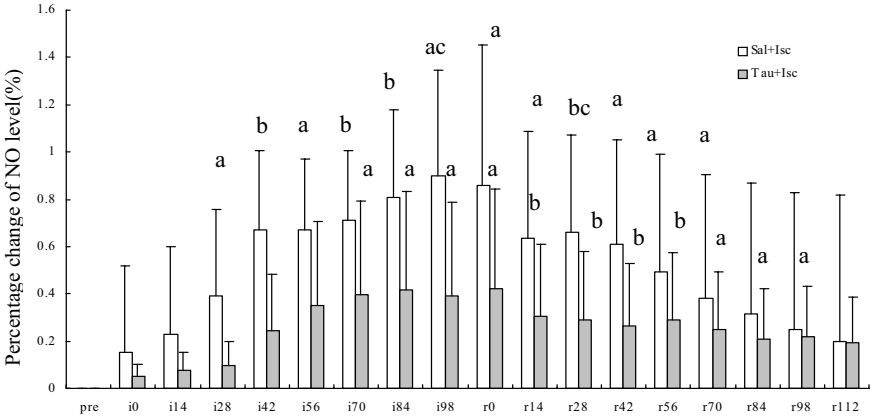

Figure 3. Change in striatum nitric oxide production during ischemia and reperfusion with or without taurine administration. Sal: saline; Tau: taurine; Isc: ischemia; Pre: before ischemia; i0: ischemia onset; i14-i98: different time points (min) after ischemia; r0: reperfusion onset; r14-r112: different time points (min) after reperfusion. a, P<0.05 *vs*. preischemia; b P<0.01 *vs*. preischemia; c P<0.05 *vs*. "Tau+Isc" group.

Fig. 3 shows the effect of taurine administration on the production of NO. After ischemia, the NO level in the dorsal lateral striatum increased rapidly and reached the peak level at 94 min post-ischemia (89.8% higher than the basal). After the onset of reperfusion, the NO level decreased gradually. As for the taurine group, a similar changing tendency was found in the NO level. The variation percentage at 98 min post-ischemia and 29 min post-reperfusion was 49% (P<0.05) and 52% (P<0.05) smaller, respectively, than that in the saline administration group.

After ischemia, heavy immunopositive staining of Bax and P53 was found in the ischemic core and penumbra. Bcl-2 immunoreactivity appeared generally in the penumbra, but could hardly be found in the core. As shown in Fig. 4, when the expression ratio of P53 (immunopositive cell number/residual cell number) and Bcl-2/Bax are compared, both EA treatment and taurine administration could notably elevate the expression ratio of Bcl-2/Bax and reduce that of P53. There was no significant difference between the two groups. However, when EA was given in combination with taurine, the Bcl-2/Bax ratio was higher and the P53 expression ratio lower than in the case of EA or taurine alone (P<0.05).

4. DISCUSSION

It has been demonstrated that taurine protects neural cells against cell-damaging conditions such as hypoxia, ischemia, free radicals and oxidative stress (Schurr *et al*., 1987; Boldyrev *et al*., 1999; O'Byrne *et al*., 2000). Our results provide further support to this notion. In the present study, 3% β-alanine in drinking water for 9 days had no obvious neurotoxicity in normal rats but significantly impaired the anti-ischemic efficacy of EA. Thus, the abatement of EA anti-ischemic efficacy possibly resulted from the

exhaustion of brain taurine. In addition, the present study also showed that taurine administration could enhance the neuroprotective effect of EA. These results indicate that

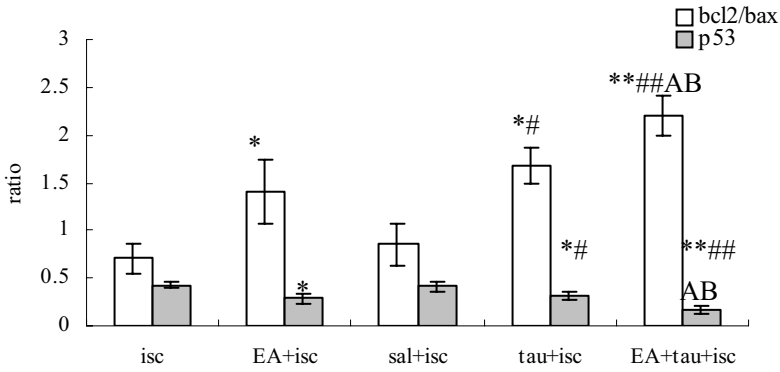

Figure 4. Expression ratio of Bcl-2/Bax and P53 in the ipsilateral cortex at 24 h post-reperfusion with taurine administration or EA treatment. isc: ischemia; EA: electroacupuncture application; sal: saline administration; tau: taurine administration. * P<0.05 compared with ischemic group; ** P<0.01 compared with ischemic group; # P<0.05 compared with "sal + isc" group; ## P<0.01 compared with "sal + isc" group; A, P<0.05 compared with "EA + isc" group; B, P<0.05 compared with "tau + isc" group.

an elevated interstitial taurine level might be one of the important mediators in the neuroprotective efficacy of EA. In the present study, the attenuation of NO production and the regulation of apoptotic-related protein expression in the taurine administration group are similar to those after EA treatment. The anti-NO effect of taurine is possibly due to its ability to inhibit iNOS mRNA expression (Redmond, 1996; Gurujeyalakshmi *et al*., 2000; Barua *et al*., 2001). EA also showed similar effects on NO system in the CNS as reported by us earlier (Zhao *et al*., 1999). These results imply that there might be some common neuroprotection pathways between taurine and EA.

In conclusion, both taurine and EA could protect the brain against ischemic injury. Taurine may play an important role in the EA anti-ischemic treatment.

5. ACKNOWLEDGMENT

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MECHANISMS OF LONG-LASTING ENHANCEMENT OF CORTICOSTRIATAL NEUROTRANSMISSION BY TAURINE

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1. INTRODUCTION

Taurine, a major organic osmolyte and neuromodulator (Huxtable, 1992; Oja and Saransaari, 1996) is ubiquitous in the brain with the highest levels in the cortical structures and basal ganglia (Palkovits *et al*., 1986). Taurine is released intensely under cell-damaging conditions (ischemia, hypoxia, metabolic poisons and others) and is thought to participate in neuroprotection due to its neuroinhibitory actions (Saransaari and Oja, 2000). In fact, in different brain structures extracellular taurine inhibits neuronal discharges via activation of Cl^- conductance associated with $GABA_A$ and strychnine-sensitive glycine receptors (Haas and Hösli, 1973; Hussy *et al*., 1997; Sergeeva and Haas, 2001). However, exposure to high extracellular taurine initiates long-lasting potentiation of glutamatergic neurotransmission in the hippocampus (Galarreta *et al*., 1996) and neostriatum (Chepkova *et al*., 2002), the functional significance of which remains unclear.

In the neostriatum, a core structure of the basal ganglia involved in motor and cognitive brain functions (Graybiel *et al*., 1994) taurine is accumulated and released by its principal cells, medium-sized spiny neurons (Clarke *et al*., 1983; Della Corte *et al*., 1987). These cells receive glutamaterigc inputs from the neocortex and thalamus (Smith and Bolam, 1990) and process information under the strict control of striatal cholinergic interneurons and dopaminergic input from the substania nigra (Calabresi *et al*., 2000). The modulation of synaptic transmission in pathological states associated with intense release and redistribution of taurine as an organic osmolyte may contribute to the disturbances of fine motor and cognitive functions in these conditions.

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Taurine 6
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Recent studies of the mechanisms of hippocampal long-lasting potentiation by taurine (LLP-TAU, Del Olmo *et al*., 2000, 2003, 2004) have disclosed many similarities with electrically-induced long-term potentiation, an activity-dependent form of synaptic plasticity presumably involved in the mechanisms of development and learning (Bliss and Collingridge, 1993). Despite phenomenological similarity, hippocampal LLP-TAU and corticostriatal LLE-TAU may have different mechanisms (Sergeeva *et al*., 2003). In this report we summarize our data on the mechanisms of corticostriatal LLE-TAU obtained by using mouse brain slice preparations and a pharmacological approach.

2. MATERIALS AND METHODS

The study was carried out on horizontal brain slices prepared from adult male C57Bl/6 mice as previously described (Chepkova *et al*., 2002) with the recording of corticostriatal field potentials. To stimulate a cortical input constant-voltage pulses of 0.1 ms duration were applied via bipolar Ni/Cr stimulation electrodes placed on the white matter between the neocortex and striatum. The recording electrode, a low-resistance glass micropipette filled with the perfusion medium or 1 M NaCl was positioned within the striatum at a distance of up to 500 μm. After evaluating the input-output relations, stimulus intensity was adjusted to induce a field potential of approximately half-maximal amplitude. The standard experimental protocol included 20-30-min of control recording, 30-min perfusion with 10 mM taurine (alone or in combination with receptor/channel antagonists or enzyme inhibitors) and 60-90-min washout. Stimuli were applied every 20 sec for the entire recording period. Fifteen consecutive responses (5-min recording) were averaged to generate one data point.

Signals were amplified, digitized at 10 kHz, and stored on a PC using pClamp8 software (Axon Instruments, Foster City, CA, USA) for off-line analysis. The N2 peak of the averaged corticostriatal field potential was measured and normalized to its mean control amplitude. Three consecutive points at 50, 55 and 60 min after taurine withdrawal were averaged to measure long-lasting effects of taurine.

The drugs used were taurine, DL-2-amino-5-phosphonopentanoic acid (AP5), (-) bicuculline methiodide, strychnine hemisulphate, nifedipine, nickel chloride, mecamylamine, scopolamine hydrochloride, SCH23390, raclopride, trifluoperazine, chelerythrine, H-89, nipecotic acid, niflumic acid (all Sigma, St. Louis, MO), guanidinoethyl sulphonate (Toronto Research Chemicals), and SR-95531 (gabazine, Tocris bioscience, Northpoint, Avonmouth, UK). Taurine was prepared by dissolving in the perfusion medium with 10 mM glucose instead of 20 mM in control to maintain the constant osmolarity and applied via perfusion system. Water-soluble antagonists were dissolved in distilled water as stock solutions and stored frozen in microsamples until use. Nifedipine and H-89 were dissolved in 50% ethanol solution and kept in microsamples at -20^{o}C until use. During the experiment the microsamples were diluted with perfusion medium to a necessary concentration and applied via perfusion system 10 -15 min before taurine.

The data are expressed as means ± SEM. Statistical comparisons were made using one-way ANOVA with Dunnett's posttest, Student's *t*-test and Fisher´s exact probability test.

3. RESULTS AND DISCUSSION

3.1. Long-Lasting Enhancement of Corticostriatal Field Responses after Exposure to Taurine Depends on Activation of Glycine Receptors

Perfusion with taurine (10 mM for 30 min) caused an initial decrease followed by a persistent increase in the amplitude of the second negative peak (N_2) of a striatal field potential (Fig. 1A) reflecting postsynaptic activation of a population of striatal principal cells by cortical afferents (Malenka and Kocsis, 1988).

Taurine-evoked initial depression was suppressed by the antagonists at $GABA_A$ (gabazine, bicuculline) and glycine receptors (strychnine) indicating that the two types of inhibitory receptors contribute to taurine-induced depression of corticostriatal responses (Fig. 1B).

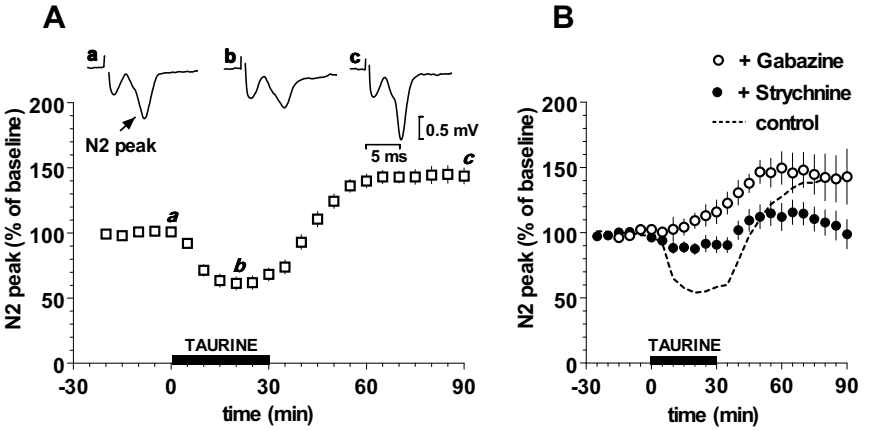

Figure 1. Long-lasting enhancement of corticostriatal field responses by taurine depends on activation of glycine receptors. (A) Changes in the amplitude of the second negative component (N2) of the corticostriatal field potential during and after perfusion with 10 mM taurine. The plot represents the mean time course of changes in 18 slices. The period of perfusion with taurine is indicated by the horizontal bar. The traces above are taken from one of the experiments summarized in the plot. Each trace is an average of 15 individual filed potentials recorded 5 min before (a), during (b), and at 55-60 min after (c) taurine application. Corresponding time periods are marked on the plot by italics. (B) The time course of N2 after application of taurine in combination with the $GABA_A$ (gabazine 10 μM, n= 4, open circles) or glycine (strychnine 10 μM, n=10, filled circles) receptor antagonists. Dotted line shows the time course of changes in control experiments (n=11).

Despite similar actions of $GABA_A$ and glycine receptor antagonists on the initial depression, they differently affect the following potentiation of corticostriatal responses. In the presence of gabazine (Fig. 1B) or bicuculline (Fig. 2A) efficiently suppressing the initial depression, LLE-TAU developed faster but did not significantly differ from the control in the peak magnitude. In contrast, the presence of strychnine decreased both the occurrence (by 50%) and the magnitude of LLE-TAU (Fig. 1B). These data show that the blockade of strychnine-sensitive glycine receptors significantly impairs the induction of LLE-TAU suggesting an important role of their activation in the mechanisms of LLE-TAU induction.

3.2. Low Voltage-Gated Calcium Channels Mediate Calcium Entry During the Induction of LLE-TAU

Many forms of synaptic plasticity are triggered by a rise in intracellular Ca^{2+} concentration regulating activity of Ca^{2+}-sensitive enzymes (Bliss and Collingridge, 1993). Calcium influx is largely provided by activation of the N-methyl-D-aspartate (NMDA) receptor channel having large Ca^{2+} permeability and voltage-gated calcium channels. We studied the role of these routes for Ca^{2+} entry in the development of LLE-TAU by using specific antagonists of NMDA receptor-coupled and voltage-gated channels. The data are summarized in Fig. 2.

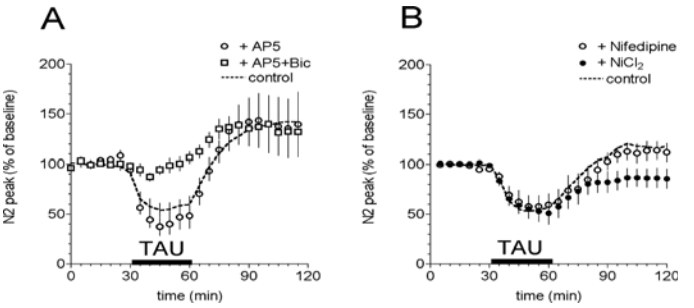

Figure 2. Development of LLE-TAU is abolished by the blockade of low voltage gated calcium channels. Plots represent the mean time course of changes in N2 amplitude. The antagonists were introduced 10 min before and 30 min together with taurine. (A) The NMDA receptor antagonist AP5 (50 μM) alone (n=5, open circles) or in combination with the $GABA_A$ receptors antagonist bicuculline (50 μM, n=6, open squares) does not affect the development of LLE-TAU. Dotted line shows the mean time of N2 changes in control experiments (n=11). (B) Nickel ($NiCl_2$, 50 μM, n=5), an antagonist of low voltage-gated calcium channels suppress the induction of LLE-TAU (filled circles), while nifedipine (10 μM, n=6), an antagonist of high voltage-gated calcium channels (open circles) does not affect LLE-TAU. Dotted line shows the mean time course of changes in the N2 amplitude in control experiments (n=6).

LLE-TAU was insensitive to the blockade of both NMDA receptors (Fig. 2A) and L-type voltage-gated channels (Fig. 2B) indicating that these routes of Ca^{2+} entry do not operate in LLE-TAU. At the same time application of taurine in the presence of Ni^{2+}, an antagonist of low voltage-activated calcium channels failed to induce LLE-TAU (Fig. 2B). This indicates that low voltage gated calcium channels (T-type) are involved in the induction of corticostriatal LLE-TAU. These findings are in keeping with previously reported results on hippocampal LLP-TAU (Del Olmo *et al*., 2000).

T-channels expressed in striatal principal neurons operate at membrane potentials between -80 and -40 mV (McRory *et al*., 2000). At 10 mM taurine can depolarize these cells by approximately 10 mV (Chepkova *et al*., 2002), sufficient for T-channels activation.

3.3. Induction of Corticostriatal LLE-TAU Is Associated with Activation of Protein Kinase C But Not Protein Kinase A

The primary Ca^{2+} sensor is calmodulin (CaM) activating a number of intracellular cascades involved in synaptic plasticity (Xia and Storm, 2005). To assess the role of some of these cascades in LLE-TAU we studied its development upon inhibition of calmodulin, protein kinase C (PKC) and A (PKA).

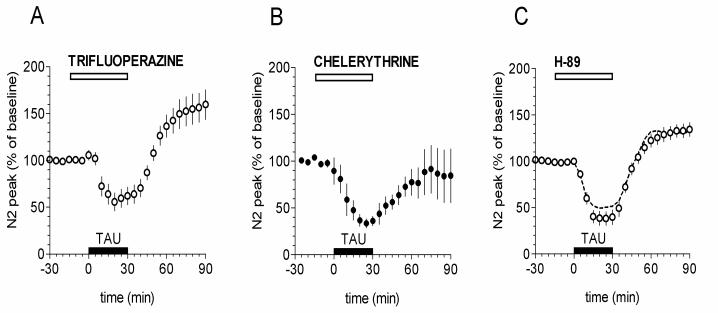

Figure 3. Activation of protein kinase C is required for the development of LLE-TAU. The plots represent the time course of changes in the amplitude of N2. All inhibitors were administered 15 min before and 30 min together with taurine. (A) Effects of trifluoperazine (10-40 μM, n=10), an inhibitor of calmodulin. (B) Effects of chelerythrine (2 μM, n=4), a specific inhibitor of protein kinase C. (C) Effects of H-89 (10 μM, n=8), an inhibitor of protein kinase A. Dotted line represents the time course of changes in the control experiments with application of vehicle (ethanol, n=8).

Bath application of the calmodulin antagonist trifluoperazine 10-15 min before and during taurine perfusion did not change either the occurrence or the magnitude of LLE-TAU (Fig. 5A). By contrast, LLE-TAU was significantly suppressed in the presence of the specific inhibitor of PKC chelerythrine (Fig. 5B). The PKA inhibitor H-89 did not affect the induction of LLE-TAU (Fig. 5C) and did not reduce its maintenance: At 80-90 min after taurine withdrawal (not shown) the magnitude of LLE-TAU remained at the same level as after 50-60 min (141 ± 6% and 142 ± 5%, respectively).

These data indicate that PKC but not PKA participates in the development of LLE-TAU and that enzymes activated by CaM (CaMKII, NOS, adenylyl cyclase) do not significantly contribute to the mechanisms of LLE-TAU.

3.4. Induction of LLE-TAU Is Controlled by Cholinergic and Dopaminergic Mechanisms

As described above, corticostriatal LLE-TAU depends on activation of glycine receptors. Strychnine-sensitive glycine receptors efficiently gated by taurine (Sergeeva and Haas, 2001) stimulate striatal release of dopamine (DA, Yadid *et al*., 1993) and acetylcholine (Ach, Beani *et al*., 1983; Taylor *et al*., 1988) indicating a presynaptic excitatory action in this brain region (Darstein *et al*., 2000). Taurine also potentiates the efflux of DA from the neostriatal tissue (Kontro and Oja, 1988; Ruotsalainen and Ahtee, 1996). Dopamine via D1 receptors excites cholinergic interneurons (Aosaki *et al*., 1998)

and thus can stimulate the release of Ach which, in its turn, stimulates DA release via presynaptic nicotinic receptors on nigrostriatal terminals (Zhou *et al*., 2002). These facts prompted us to study the role of striatal DA and Ach in the development of corticostriatal LLE-TAU.

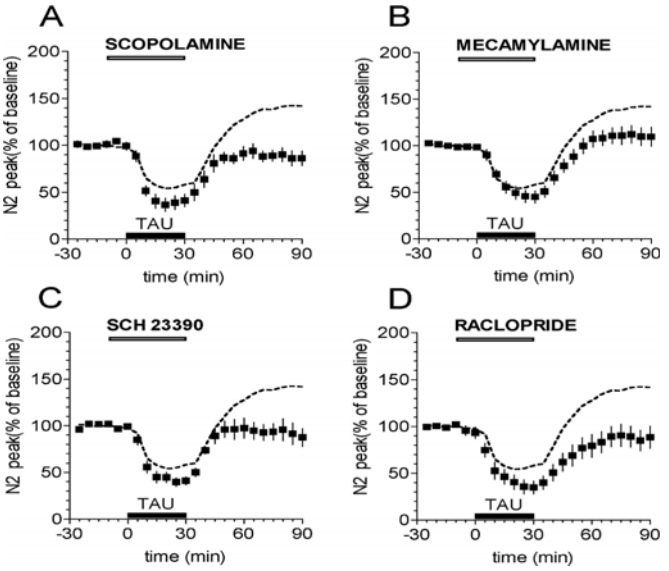

Figure 4. Cholinergic and dopamineric antagonists suppress the induction of LLE-TAU. The plots represent the mean time course of relative changes in the amplitude of the N2 component. All antagonists at 10 μM were administered 10 min before and 30 min together with taurine. (A) Effects of the muscarinic antagonist scopolamine (n=5). (B) Effects of the nicotinic antagonist mecamylamine (n=9). (C) Effects of SCH23390, a specific antagonist at D1-like dopamine receptors (n=6). (D) Effects of raclopride, a specific antagonist at D2-like dopamine receptors (n=6). Dotted line shows the mean time course of changes in control experiments.

The muscarinic antagonist scopolamine applied 10 min before taurine and for the entire period of taurine perfusion prevented the development of LLE-TAU (Fig. 4A), indicating that muscarinic cholinoceptors are critically involved in its induction. Similar application of the nicotinic antagonist mecamylamine (Fig. 4B) significantly reduced the occurrence of LLE-TAU without affecting the magnitude of enhancement in those preparations where it occurred (3 of 9).

The occurrence of LLE-TAU was also considerably reduced by the two specific antagonists of DA receptors (Fig. 4C, D). In the presence of the D1 antagonist SCH23390 or the D2 antagonist taurine was able to induce low-magnitude LLE-TAU in only 2 of 6 slices in each set of the experiments.

These data indicate that corticostriatal LLE-TAU is controlled by both dopaminergic and cholinergic mechanisms and in this respect is similar to electrically-induced LTP, an activity-dependent form of plasticity in corticostriatal input (Calabresi *et al*., 2000; Centonze *et al*., 2001).

3.5. Long-Lasting Enhancement of Corticostriatal Neurotransmission by Taurine Depends on Taurine Uptake

Taurine uptake and intracellular accumulation have been suggested as important determinants of its long-term effects on hippocampal synaptic transmission (Galarreta *et al*., 1996; del Olmo *et al*., 2000) but recent investigations of their role in the induction of hippocampal LLP-TAU produced controversial results (Sergeeva *et al*., 2003; Del Olmo *et al*., 2004; Dominy *et al*., 2004).

Neostriatal tissues express a high level of messenger RNA for a specific taurine transporter (TauT, Liu *et al*., 1992). We used pharmacological and genetic approaches to investigate the role of taurine uptake by TauT in corticostriatal LLE-TAU.

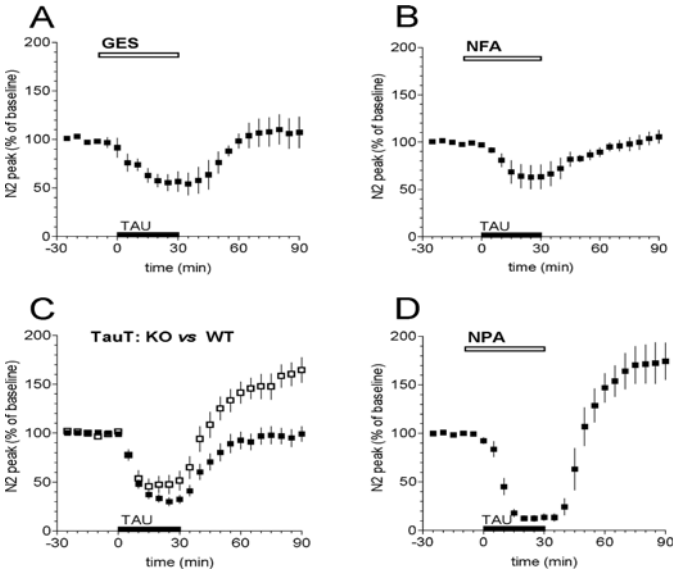

Figure 5. Experimental interventions in the activity of taurine uptake systems impair the induction of LLE-TAU. The plots represent the time course of changes in the N2 amplitude. (A) The effect of guanidinoethyl-sulphonate (GES, 1 mM, n=7), a competitive inhibitor of a high-affinity taurine uptake. (B) The effect of niflumic acid (NFA, 10 μM, n=5), a non-selective antagonist of anion channels. (C) The time course of taurine-induced changes in the N2 amplitude in brain slice preparation from mice with the knockout of a high-affinity taurine transporter (TauT KO, n=20, filled squares) and from corresponding wild type mice (WT, n=18, open squares). (D) Effects of nipecotic acid (NPA, 1 mM, n=4), a broad-spectrum inhibitor of GABA transporters.

Guanidinoethylsulphonate (GES 1 mM), a competitive inhibitor of a high affinity taurine uptake by TauT suppressed the development of LLE-TAU (Fig. 3A) when introduced into the medium 10 min before and during taurine application. These data imply that taurine uptake by TauT is involved in the induction of LLE-TAU. This conclusion was further substantiated by the results of taurine application to corticostriatal slices from taurine-deficient mice with the TauT-encoding gene knocked out (Heller-Stilb

et al., 2002). These slices showed significantly reduced ability to generate LLE-TAU (Fig. 3C): post-taurine enhancement of responses was observed in only 40% of the slice preparations *vs* 94% in slices from wild type animals.

Taurine at millimolar extracellular concentrations can be transported by low-affinity GABA transporters (GATs) and penetrate cell membranes by diffusion (Holopainen *et al*., 1983; Kontro and Oja, 1983). We examined the possible involvement of these routes of taurine entry by studying LLE-TAU development in the presence of the broad-spectrum GAT inhibitor nipecotic acid and niflumic acid (NFA), an antagonist of organic osmolyte/anion channels.

Niflumic acid at a relatively low concentration (10 μM) practically abolished LLE-TAU and delayed the recovery of field potentials (Fig. 4B). Nipecotic acid exerted no such effects although it considerably increased the initial depression of responses (Fig. 4D) which could result from extracellular GABA accumulation (Solis and Nicoll, 1992)

Thus, interventions in taurine active transport (TauT) or passive accumulation (NFA-sensitive anion channels) significantly impaired the development of LLE-TAU indicating the necessity of taurine uptake for its induction.

4. SUMMARY AND CONCLUSIONS

The long-lasting enhancement of corticostriatal neurotransmission by taurine, LLE-TAU represents a complex phenomenon requiring concurrent activation of glycine, DA and Ach receptors as well as taurine uptake.

The data on the mechanisms of corticostriatal LLE-TAU can be integrated in the following scheme. Taurine interaction with glycine and $GABA_A$ receptors causes depolarization of striatal medium spiny cells (Chepkova *et al*., 2002) which is enhanced by taurine electrogenic uptake by TauT (Sarkar *et al*., 2003). This depolarization leads to Ca^{2+} entry via low voltage gated Ca^{2+} channels. Muscarinic M1 receptors are expressed in medium spiny neurons (Yan *et al*., 2001) and regulate their excitability mostly via phospholipase C (PLC)/PKC cascade (Lin *et al*., 2004). Concurrent activation of M1 and PLC-coupled D1 receptors (O'Sullivan *et al*., 2004) can amplify Ca^{2+} signal via IP3-stimulated Ca^{2+} release from intracellular stores and stimulate PKC.

5. ACKNOWLEDGMENTS

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INCREASED GAD-POSITIVE NEURONS IN THE CORTEX OF TAURINE-FED MICE

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1. INTRODUCTION

During early critical periods of developmental maturation, the brain is very sensitive to environmental factors. In this study, we supplemented mice with taurine in drinking water for four weeks and examined changes in the inhibitory system. After four weeks treatment with taurine, mice showed increased levels of the inhibitory neurotransmitter GABA and its synthesizing enzyme, glutamate decarboxylase (GAD), Indicating that chronic taurine treatment induces biochemical changes to the inhibitory GABAergic system. Previously, we showed an increase in both isoforms of GAD using western blots. Here we tested if there was an increase in the number of GAD positive neurons in the brain.

To better elucidate how specific interneuronal subtypes contribute to both normal and pathological brain functions, it is essential that these neurons be easily identifiable during experimental manipulations. To facilitate the study of GABAergic inhibitory interneurons, we used transgenic mice that selectively express the enhanced derivative of the auto-fluorescent protein, green fluorescent protein (EGFP) in a subpopulation of GABAergic neurons. In these mice an upstream regulatory region from the murine *Gad1* gene, which codes for the 67 kDa form of the GABA synthesizing enzyme, GAD, was used to drive EGFP expression, as this gene appears to be ubiquitously expressed in GABAergic neurons. Thus, by examining the pattern of EGFP expression, we can directly observe changes that occur in GABAergic neurons, which will serve to validate the biochemical observations and will allow us to determine the type of neuro-architectural changes (such as changes in the number of inhibitory interneurons or changes in their dendritic morphology) that occur in the brain. We found that, consistent with the increased expression of GAD, there was an increase in the number of EGFP-GAD positive neurons in the cortex of taurine-fed mice. This increase in GAD expression would be a

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compensatory mechanism to the increased excitability observed under chronic treatment with taurine, as shown by increased vulnerability to kainic acid-induced seizures.

2. MATERIAL AND METHODS

2.1. Drug Administration

Taurine was provided in drinking water (0.05% w/v) for one month before perfusing the mice and collecting the brain for analysis. All mice used in these experiments were two months old and supplementation of taurine in drinking water started at four weeks of age.

2.2. Screening of Transgenic Mice

Transgenic mice were screened via PCR similar to the method described by Busler and Li (1996). Briefly, toe clips were placed in a lysis buffer (20 mM Tris HCl, pH 8.4, 50 mM KCl, 1 mM EDTA, pH 8.0, 0.1 mg/ml gelatin, 0.1% Triton X-100, and 4 mg/ml proteinase K) at 55°C for 3-5 hr, then 96°C for 10 min, then placed on ice, and 5 µl of the cold lysate used directly for PCR (100 µl reaction volumes). Each PCR reaction used a transgene-specific primer set and a primer set that spanned intron 15 of the murine *Gad1* gene (Bu and Tobin, 1994; Yanagawa *et al.,* 1997).

2.3. Counting EGFP-Expressing Neurons

EGFP transgenic mice were anesthetized with Nembutal and perfused transcardially with 3 ml of PBS followed by 20 ml of 4% para-formaldehyde. After post-fixation *in situ* overnight at 4^{o}C in 4% para-formaldehyde, brains were removed, cryoprotected in 30 % sucrose and frozen at -80^{o}C. Coronal cryosections (30 µm) were collected in a 12 well plate containing cryoprotectant (30% glycerol, 30% ethyleneglycol and 40% PBS). After serially cutting all the brain, the content of the first and seventh well were the first and the seventh section of each round was directly mounted on a gelatin-coated slide. This arrangement produced sections separated by 180 µm spanning the whole brain. Sections were allowed to dry overnight at 4^{o}C in a slide box. Subsequently sections were stained with a fluorescent red Nissl stain to facilitate identification of brain structures and cover slips were mounted. Images were captured at low magnification (highest field of view) with a Zeiss Axioskop2 microscope equipped with AxioCam MRc5 digital camera. Images were then transferred to Adobe Photoshop 6.0 and the number of EGFP-expressing neurons in each image was counted.

3. RESULTS

3.1 Pattern of EGFP Expression in the Brain

To better elucidate how specific interneuronal subtypes contribute to both normal and pathological brain functions, it is essential that these neurons be easily identifiable during experimental manipulations. To facilitate the study of GABAergic inhibitory interneurons,

we are using transgenic mice that selectively express the enhanced derivative of the auto-fluorescent protein, green fluorescent protein (EGFP) in a subpopulation of GABAergic neurons. In these mice an upstream regulatory region from the murine *Gad1* gene (Oliva *et al.,* 2000), which codes for the 67 kDa form of the GABA synthesizing enzyme, GAD, was used to drive EGFP expression, as this gene appears to be ubiquitously expressed in GABAergic neurons. Thus, by examining the pattern of EGFP expression, we can directly observe changes that occur in GABAergic neurons, which will serve to validate the biochemical observations and will allow us to determine the type of neuro-architectural changes (such as changes in the number of inhibitory interneurons or changes in their dendritic morphology) that occur in response to hypersensitivity. In brain sections prepared from these mice, EGFP appeared to freely diffuse throughout the cytoplasm of expressing neurons (Fig. 1). In fixed preparations, the overwhelming majority of EGFP-expressing neurons were very intensely fluorescent, making their processes readily visible and traceable to their terminations. In most instances, neuronal processes could be visualized and followed for hundreds of micrometers from their parent somata. In these mice, induction of hippocampal and cortical EGFP expression was found to begin at approximately postnatal day 5 (Oliva *el al.,* 2000). This developmental onset of EGFP expression temporally coincides with the terminal differentiation of GABAergic interneurons and the onset of expression of many of the macromolecules (e.g., somatostatin) that elineate subpopulations of mature GABAergic neurons (Naus *et al.,*

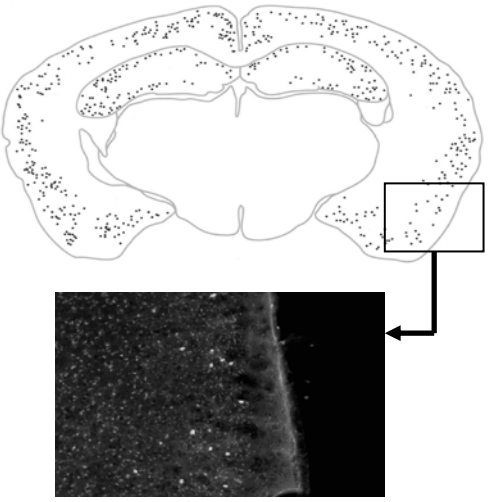

Figure 1. Pattern of EGFP expression found in the brain of two-month-old EGFP transgenic mice. This image is of a 30-µm-thick coronal brain section from a homozygotic EGFP transgenic mouse. All sections were stained with a red fluorescent Nissl stain (Molecular Probes) to facilitate visualization of neuronal structures. Images were captured with Zeiss Axioskop2 microscope equipped with AxioCam MRc5 digital camera. Photomicrograph illustrating the pattern of EGFP in the somatosensory cortex. The image was digitally inverted. This expression pattern typifies the one seen in all cortical areas. Laminar specificity of EGFP expression demonstrated in primary cortex from a 30-µm-thick section. EGFP-expressing somata are restricted mainly to layers II-IV and upper layer V. Magnifications 10X objective.

1988; Bergmann *et al.,* 1991; Jiang and Swann, 1997). To ascertain the identities of EGFP-expressing neurons in these transgenic mice, fluorescence immunohistochemistry for numerous markers of GABAergic interneurons was performed (Oliva *el al.,* 2000).

3.2. Taurine-Fed Mice Have Elevated EGFP Expression in the Brain

To test if taurine treatment affected the number of GAD-, and thus, EGFP-positive neurons, we supplemented taurine (0.05%) in the drinking water for four weeks and counted the number of EGFP-positive neurons in the cortex of these mice. We counted the number of EGFP-positive neurons in approximately 100 coronal sections from each mouse comprising the parieto-temporal lobes of the cortex. We used for this study four pairs of taurine-fed and age-matched controls (siblings). Fig. 2 shows a representative comparison between a taurine-fed and a control mouse brain. Each data point on the graph represents the number of EGFP-positive neurons in the cortex per coronal section with section 1 is the most rostral to 100 the most caudal in the study.

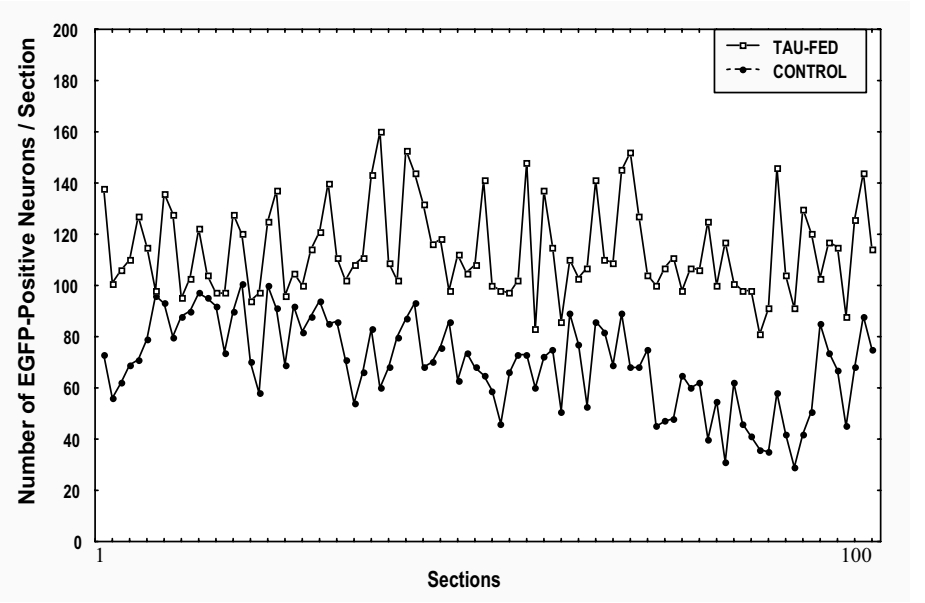

Figure 2. Coronal sections for analysis of EGFP expression encompass the parieto-temporal cortex indicated by the arrow and consisted of 100 serial coronal sections along the rostro-caudal axis. Line graph is a representative comparison between brains of a control and taurine-fed mouse.

A quantification of the number of EGFP-positive neurons in all the sections examined from both taurine-fed and controls showed that the taurine-fed mice have a significant increase in the number of EGFP-positive neurons in the cortex (Fig. 3). This is consistent with previous observations where we found an increase in the expression levels of GAD in the brains of taurine-fed mice when compared to age-matched controls.

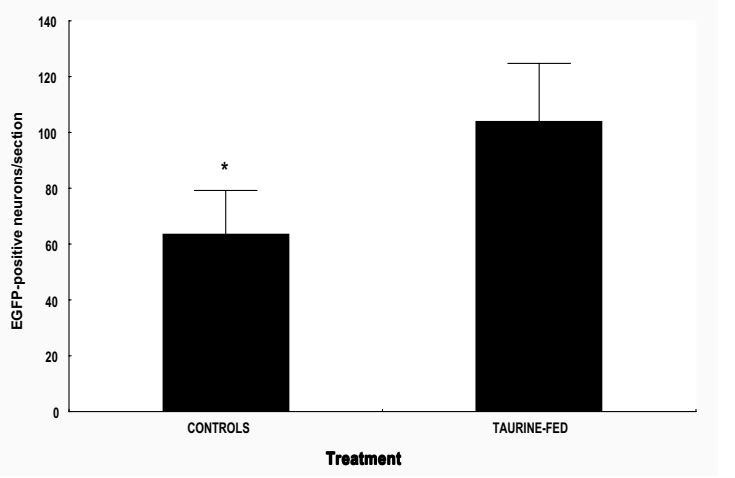

Figure 3. Taurine induces an increase in the number of EGFP-positive neurons. Bars represent the mean ± SD of EGFP-positive neurons obtained from four controls and four taurine-fed mice (n ≈ 400, 100 sections per brain). The increase in the number of EGFP-positive neurons in the taurine-fed mice was significant ($p < .05$).

4. DISCUSSION

In the adult brain, the excitability of neuronal circuits is controlled by inhibitory GABAergic interneurons. In this study, we supplemented taurine in drinking water (0.05 %) for 4 continuous weeks and tested the effects on neuronal excitability. We found that taurine-fed mice showed increased susceptibility to KA-induced seizures (El Idrissi *et al.,* 2003). Associated with this increased state of brain excitability, we found some biochemical changes in the GABAergic system. Chronic treatment with taurine in drinking water caused an increase in the levels of glutamate and GABA as well as the enzyme responsible for GABA synthesis, glutamate decarboxylase (GAD). We also found a reduced hippocampal expression of the β subunit of $GABA_A$ receptors.

To further investigate these biochemical changes in the GABAergic system, we used transgenic mice that selectively express the enhanced derivative of the auto-fluorescent protein, green fluorescent protein (EGFP) in a subpopulation of GABAergic neurons. In these mice an upstream regulatory region from the murine *Gad1*gene (Oliva *el al.,* 2000), which codes for the 67 kDa form of the GABA synthesizing enzyme, GAD, was used to drive EGFP expression, as this gene appears to be ubiquitously expressed in GABAergic neurons. Thus, by examining the pattern of EGFP expression, we can directly observe changes that occur in GABAergic neurons, which will serve to validate the biochemical observations and will allow us to determine the type of neuro-architectural changes that occur in response to hypersensitivity. In brain sections prepared from these mice, EGFP appeared to freely diffuse throughout the cytoplasm of expressing neurons (Fig. 1). In fixed preparations, the overwhelming majority of EGFP-expressing neurons were very intensely fluorescent, making their processes readily visible and traceable to their terminations. In most instances, neuronal processes could be visualized and followed for

hundreds of micrometers from their parent somata. In these mice, induction of hippocampal and cortical EGFP expression was found to begin at approximately postnatal day 5 (Oliva *el al.,* 2000). This developmental onset of EGFP expression temporally coincides with the terminal differentiation of GABAergic interneurons and the onset of expression of many of the macromolecules (e.g., somatostatin) that delineate subpopulations of mature GABAergic neurons (Naus *et al.,* 1988; Bergmann *et al.,* 1991; Jiang and Swann, 1997). To ascertain the identities of EGFP-expressing neurons in these transgenic mice, fluorescence immunohistochemistry for numerous markers of GABAergic interneurons was performed (Oliva *el al.,* 2000).

GAD, which is responsible for GABA synthesis in GABAergic neurons, has two isoforms, 65 and 67 kDa (GAD65 and GAD67), encoded by different genes (Erlander *et al.,* 1991). The expression of both isoforms has been shown to be activity-dependent (Nishimura *et al.,* 2001; Ramirez and Gutierrez, 2001) and to be influenced by the effectiveness of GABAergic inhibition (Ribak *et al.,* 1988; 1993). Since reduced $GABA_A$ receptor expression would increase excitability, the increased GAD expression could be a compensatory mechanism for reduced efficacy of the inhibitory system. This is particularly interesting because increased GAD can be a compensatory response to the increased excitability (Ramirez and Gutierrez, 2001, El Idrissi *et al.,* 2004) that would be the net result of decreased GABAergic inhibition.

We suggest that taurine-fed mice have elevated extracellular taurine levels, which would lead to sustained activation or at least binding to $GABA_A$ receptors. Such a chronic interaction of taurine with $GABA_A$ receptors may lead to down-regulation of $GABA_A$ receptor function or expression. In response to these changes, there is increased synthesis of GABA by GABAergic neurons, as compensatory mechanism to reduced post-synaptic inhibition. Furthermore, we found an increase in the number of GAD-positive neurons. This suggest that neuronal plasticity in this system is not limited to the actual inhibitory synapses where there is decrease receptor expression on postsynaptic membranes and increase neurotransmitter synthesis on the presynaptic side but rather the entire system compensate for this state of excitability by up-regulating the number of inhibitory interneurons.

It has been shown that the degradation of cerebral cortical function during old age is due to the significant age-related loss of the GABAergic function (Leventhal *et al.,* 2003). Leventhal and colleagues (2003) were able to show pharmacologic reversal of the age-related loss of orientation and directional selectivity by iontophoretic application of GABA or muscimol in aged primate visual cortical neurons *in vivo*. Gleich *et al.* (2003) improved behavioral measures of temporal coding in young adult gerbils which displayed slowed temporal processing by pharmacologically increasing GABA levels. The findings of the current study reinforce the role of GABA inhibition in the maintenance of functional neuronal circuits characterized by a critical balance between excitatory and inhibitory inputs and may have important implications for the treatment of the sensory, motor, and cognitive declines that accompany old age.

5. ACKNOWLEDGMENTS

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TAURINE CONCENTRATION IN HUMAN GLIOMAS AND MENINGIOMAS: TUMORAL, PERITUMORAL, AND EXTRATUMORAL TISSUE

Suzana Cubillos[1], Francisco Obregón[1], María Fernanda Vargas[2], Luis Antonio Salazar[2], and Lucimey Lima[1,2]

1. ABSTRACT

Taurine concentrations were determined in gliomas from 16 patients and in meningiomas from 15 patients. After imaging analysis and clinical evaluation to consider the level of functional deterioration by the scale of Karnosky, tissue was obtained by surgery. Tumoral, peritumoral and extratumoral samples were taken and analyzed by HPLC with fluorescence detector. The concentration of taurine (nmol/mg protein) was higher in tumoral and peritumoral tissues than in the extratumoral samples for gliomas. In the case of meningiomas, the taurine concentration was higher in tumoral than in peritumoral and extratumoral samples. These modifications might be due to specific functions of this amino acid, being either protective or involved in the proliferation of cells. The differential distribution in the two types of tumors could be related to the malignancy of them, which is higher in gliomas than in meningiomas.

2. INTRODUCTION

Taurine is involved in several central nervous system neurophysiological processes, involving stability of membranes, modification of calcium fluxes, and regulation of neuronal excitability. It also favors regeneration or behaves as an osmoregulator, among other functions (Huxtable, 1992; Lima *et al.,* 2001; Militante and Lombardini 2002). Extracellular taurine, determined by intraoperative dialysis, is increased in malignant gliomas, which increase is correlated to the stage of cell proliferation and to the presence of edema (De Micheli *et al.,* 2000). In addition, an increase in amino acids has been reported in these tumors by spectroscopy nuclear magnetic resonance and positron emission tomography (Schober *et al.,* 1985; Kawai *et al.,* 1991). The differential signal of

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glutamine, for instance, seems to be useful in differentiation of pathological grades of astrocytomas (Pascual *et al.,* 1998). In other type of tumors, such as colon carcinoma (Moreno *et al.,* 1993) and murine fibrosarcoma induced by radiation (Evanochko *et al.,* 1984), elevated taurine also occurs. In the first particular study it was proposed that the high levels of taurine could be related to cell proliferation and tumor malignancy (Moreno *et al.,* 1993). The aim of this study was to quantify and to compare the levels of taurine in gliomas and meningiomas, brain tumors with differential malignancy, by analyzing tumoral, peritumoral and extratumoral tissues.

3. MATERIALS AND METHODS

The patients with gliomas were 16 (14-67 years, 10 men) and with meningiomas, 15 (24-66 years, 6 men). Karnosky scale (1949, 0 to 100), for determining functional conditions of the patients, was lower than 60, indicating low deterioration. All patients had previous image studies, such as computerized axial tomography and nuclear magnetic resonance. Histopathological diagnosis was also made. The samples were obtained during surgery and homogenized in 20% sulfosalicylic acid according to their weight. Taurine was determined by HPLC with fluorescent detector and derivatization with *o*-pthalaldehide (Lima *et al.,* 1989). Proteins were determined by the method of Lowry *et al.* (1952), and the results expressed as nmol/mg of protein. Statistical analysis was performed with the use of GraphPad (Prisma, 2003).

4. RESULTS

In gliomas, the taurine concentrations (mean ± SEM) were 4.22 ± 0.82; 3.29 ± 0.48; and 1.60 ± 0.33 in tumoral, peritumoral and extratumoral tissues, respectively. In meningiomas, the taurine concentrations (mean ± SEM) were 8.82 ± 2.06; 3.35 ± 0.58; and 2.30 ± 0.45 in tumoral, peritumoral and extratumoral tissues, respectively (Fig. 1).

5. DISCUSSION

The elevation of taurine in the core of the tumors could be related to the increase in vascular tissue or to its protective role as osmoregulator facing the injury (Oja and Saransaari, 1996), although the great increase in taurine in the tumoral tissue of both types of tumors might be related to cell proliferation or to the need of protection in the center of the malignant area. The fact that there was no difference between the tumoral and the peritumoral tissue in the case of gliomas could be an index of the differential behavior of gliomas and meningiomas, showing an abrupt gradient when comparing peritumoral and extratumoral tissue in meningiomas, which are less malignant. The observation that taurine concentrations were significantly reduced in peritumoral tissues as compared to gliomas, and not different from extratumoral tissue in the case of meningiomas, may be an indication of relation between taurine levels and malignancy.

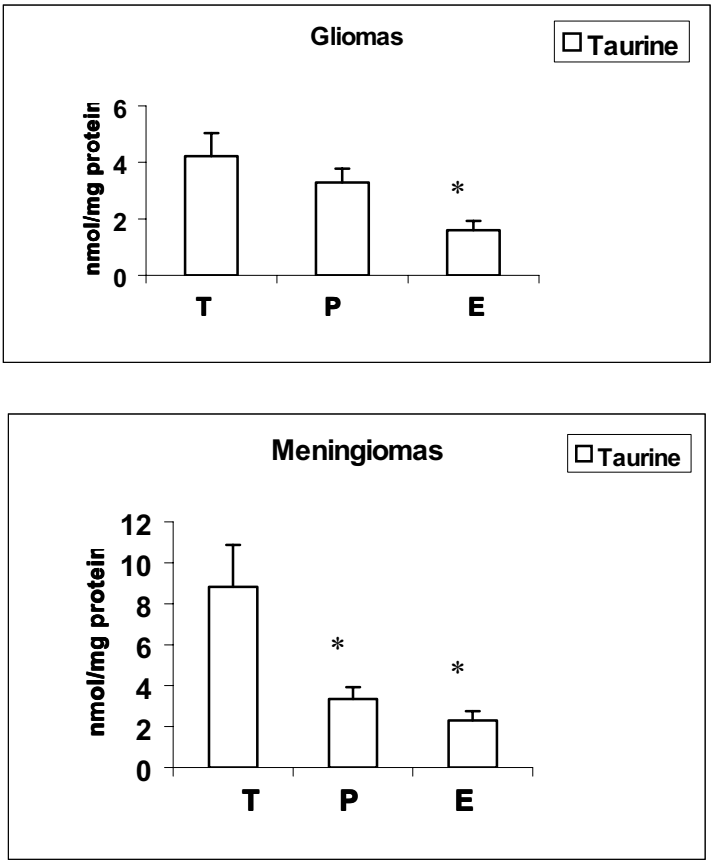

Figure 1. Taurine concentrations in T tumoral, P peritumoral; and E extratumoral tissues. * P<0.05, for differences with respect to T, or T and P.

The variations between the samples and the tumors indicate specific modulation and possible roles of taurine in brain tumors. In addition, we could also speculate that the presence of high taurine in the center of the tumors could be playing a protective effect, even modulating electrical activity, although glutamate and gamma-aminobutyric acid are lower in tumoral than in extratumoral tissue (data not shown). Further studies are focused in understanding role of taurine in brain tumors.

6. ACKNOWLEDGMENTS

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TAURINE TRANSPORT AND TRANSPORTER LOCALIZATION IN PERIPHERAL BLOOD LYMPHOCYTES OF CONTROLS AND MAJOR DEPRESSION PATIENTS

Fili Fazzino[1], Mary Urbina[1], Salvador Mata[2], and Lucimey Lima[1,2]

1. ABSTRACT

Taurine concentration is increased in peripheral blood lymphocytes in a group of depressed subjects. After this observation [^{3}H]taurine transport was measured in lymphocytes of 10 major depression patients (19-60 years old), diagnosed by the criteria of the American Psychiatric Association, with moderate severity as determined by Hamilton Scale for Depression (32 ± 6). The control group comprised 10 subjects (20-56 years old). Taurine transporter and CD4+ (helpers) and CD8+ (cytolytic) T lymphocytes were immunolabeled. No significant differences were observed in immunochemical analyses. CD4+ cells were 53% and CD8+ cells 28% of the total lymphocytes in both controls and patients. Two taurine transport components, high and low affinity, were demonstrated in both groups. Taurine transporter was present in 16% of CD4+ and CD8+ lymphocytes in controls and patients. This preliminary report exhibits the presence of taurine transporter in peripheral blood lymphocytes but no differences were observed between controls and depressed subjects.

2. INTRODUCTION

We previously demonstrated that in blood peripheral lymphocytes of major depression patients there was an increase in taurine levels of about 50% (Lima *et al.,* 2003). Moreover, the treatment with the antidepressant mirtazapine reduced this elevation to control values. In that particular report we also showed the increase in aspartic acid and glutamine, but gamma-aminobutyric and glutamic acids were not altered. In addition,

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the levels of taurine in lymphocytes correlated significantly and positively with the severity of depression as measured with Hamilton Scale for Depression (HAM-D, Hamilton, 1960). Some authors consider that taurine in white blood cells probably protects these cells against oxidative stress (Lubec *et al.,* 1996) or it could be related to regulation of osmolarity by being released (Pasantes-Morales *et al*., 1991). Chick embryo B lymphocytes possess a taurine transport system whose function is related to development (Porter and Martin, 1992). Due to the above observations, we did now a preliminary approach to study taurine transport in peripheral blood lymphocytes.

3. MATERIALS AND METHODS

Ten patients (19 to 60 years old) were diagnosed following the DSM-IV criteria for major depression with the Structured Clinical Interview for Disorders of Axis I (SCID-I) (American Psychiatric Association, 1994). All patients gave their written informed consent. They did not have other major psychiatric disorder or somatic illness, did not evidence suicidal risk factors, and should be free of drugs at least two weeks prior to taking blood samples and initiating antidepressant treatment. HAM-D was used for determining the severity of depression, minimal score of 18. Control group (10, 20-56 years old) was evaluated by the psychiatrist to discard mental disease, and they did not have a family history of psychiatric illnesses. None of the patients or controls consumed large quantities of caffeine or was suspected of drug or alcohol abuse. Blood samples were taken by venipuncture (40 ml + 0.6 ml of heparine, 1000 units/ml). The isolation of lymphocytes (80 to 90% CD3+, << 1% platelets) was done by density gradients in Ficoll/Hypaque (1077 g/l) and differential adhesion to plastic, as previously described (Urbina *et al*., 1999).

The transport of [^{3}H]taurine (32 kCi/mol) into lymphocytes was standardized by using various concentrations of lymphocytes, time of pre-incubation and incubation, and variable amounts of taurine for isotopic dilutions. Number of cells was set in 300,000 per tube, pre-incubation was 2 min and incubation 5 min, initiated by the addition of the amino acid and terminated by rapid filtration. The concentrations of taurine were from 0.001 to 5 mM. Immunolabeling for taurine transporter was done in lymphocytes fixed in formaldehyde with a rabbit anti-rat polyclonal antibody, 1:50 from the original preparation (Pow *et al.,* 2002) and an anti-rabbit IgG conjugated with fluoresceine (1:100). For identification of CD4+ (helpers) and CD8+ (cytolytic) lymphocytes commercials antibodies, mouse anti-human (1:20), were used and the second antibody was anti-mouse IgG conjugated with rodamine (1:100) (Santa Cruz). Incubation with first antibodies was done overnight at 4^{o}C and with the second for 60 min at room temperature. Samples were observed in a Nikon microscope and cells were counted for determining localization and co-localization of the markers.

4. RESULTS

Score for severity of depression by HAM-D was 32 ± 6 (mean ± SEM), which correspond to moderate. The uptake of taurine was saturable, and best fitted to two components: r square 0.92. There was no significant difference between the two groups of subjects with V_{max} in controls 0.093 and 0.225 and in patients 0.120 and 0.294

fmol/10^6 cells, high and low affinity components, respectively. K_t: was in controls 4 and 139 μM and in patients 4 and 152, respectively. Controls presented 15.63 ± 2.13 and depressed 15.04 ± 1.88 of positive cells for taurine transporter (Figs. 1A,B). The percentage of CD4+ cells in lymphocytes was 53% and that of CD8+ cells 28% in both controls and patients. Taurine transporter was present in 16% of CD4+ and CD8+ lymphocytes in controls and patients.

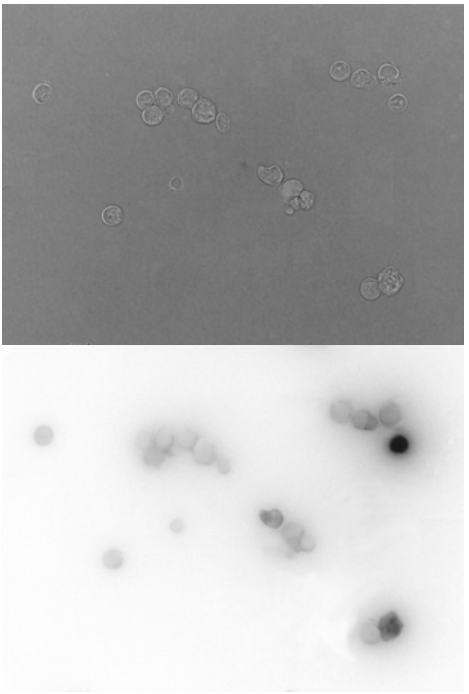

Figure 1. (A) Lymphocytes in light microscope. (B) Fluorescent lymphocytes in black, expressing taurine transporter. 1 mm = 20 μm.

5. DISCUSSION

These results indicate that, in this particular group of patients, there were no significant differences concerning taurine transport or number of lymphocytes expressing the transporter. Thus, the increase in taurine concentration occurring in a group of depressed patients (Lima *et al.,* 2003) might be explained by other mechanisms. However, the present results demonstrate the differential distribution of taurine transporter in CD4+ and CD8+, it being more co-localized with CD8+ cells, as it was observed for serotonin transporter (Montes, 2004). Taurine might exert several functions in cells from the immune system. For instance, lymphocytes express receptors for several neurotransmitters, including amino acids such as glutamate (Poulipoulou *et al.,* 2005). The fact that they contain taurine could be relevant for its functions as a modulator of cell

activity. Taurine is also released from Jurkat T-lymphocytes, stimulated by caspases, but not necessarily related to apoptosis (Lang *et al.,* 2000). In addition, taurine and alanine blocks death of bovine lymphocytes produced by high temperature (Malayer *et al.,* 1992), and taurine improves proliferative response in old T cells from mice by a calcium-mediated mechanism (Negoro and Hara, 1992).

The modifications of taurine in peripheral blood lymphocytes from major depression patients have not been addressed. It is thus poorly understood at present. In this type of study, a larger number of subjects will be important for statistical comparisons and sustained conclusions.

6. ACKNOWLEDGMENTS

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RETINA AND OPTIC TECTUM INTERACT TO MODULATE TAURINE EFFECT ON GOLDFISH AND RAT RETINAL EXPLANTS OUTGROWTH

Lucimey Lima* and Suzana Cubillos

1. ABSTRACT

The medium from cultured optic tectum of the goldfish intact or at various days after optic nerve crush, or the co-culture of this optic tectum with post-crush goldfish retinal explants plating in the absence of fetal calf serum, but in the presence of glucose, modulate its outgrowth. The addition of taurine did not further stimulate outgrowth but rather inhibited it in the presence of optic tectum. These processes were related to calcium fluxes and taurine transport into the cells. Taurine increased the length of neurites from 5-day-old rat retinal explants in the presence of fetal calf serum. The goldfish optic tectum, either medium or in co-culture with the retina, stimulated retinal outgrowth. The study of optic nerve regeneration in the presence of defined media contributes to understanding tissue-target and interspecies interaction.

2. INTRODUCTION

The post-crushed optic nerve results in regeneration of goldfish retinal explants cultured in the presence of fetal calf serum (FCS), and taurine exerts trophic effects in this system (Lima *et al*., 1988; Lima, 1999). In the absence of FCS, but in the presence of glucose, outgrowth still occurs and is stimulated by taurine (Cubillos *et al*., 2002).

Conditioned media from aggregates or explants of retinal cells increase survival of ganglion cells of newborn rats (de Araujo and Linden, 1990), indicating the relevance of target-independent processes. However, structures such as the retina and visual midbrain seem to be determinants during chicken tectal neuronal development, which occurs in an independent manner from the retina, but being dependent once synaptic contacts are made (Luksch and Poll, 2002). In addition, the brain-derived neurotrophic factor and

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neurotrophins 4/5 constitute anterograde survival molecules during development in the superior colliculus of the rat (Spalding *et al*., 2002). It has been known that trophic factors influencing visual system formation or its regeneration are produced in the retina, the target, and the optic nerve, involving neurons and glial cells (Schwalb *et al*., 1995; Cubillos *et al*., 2002;). The target regulates retino-tectal projections in the goldfish (Murray *et al*., 1982; Cook, 1990). For instance, regeneration of the optic nerve enhances cell proliferation in the optic tectum (Stevenson and Yoon, 1978), and increases biochemical activity (Benowitz and Lewis, 1983; Melzer and Powers, 2001).

Thus, a lesion of the optic nerve impairs the communication with the retina and triggers changes in the target that could modulate outgrowth from the retina. With this basis the aims of the present work were to detect possible influences of substances produced by the optic tectum of the goldfish on goldfish and rat retinal explants, and to study the interaction with taurine.

3. MATERIALS AND METHODS

3.1. Animals and Retinal Cultures With Medium From Optic Tectum or in Retino-Tectal Co-culture

The optic nerve of goldfish (*Carassius auratus*) 5-6 cm was crushed with fine forceps. The optic tectum was dissected according to Beltramo *et al*. (1994), either intact (NLOT) or at various days after the crush of the optic nerve (LOT) at 1, 3, 5, 10, 14, or 20 days. The tectum was plated in Leibovitz medium, L-15 with 0.1 mg/ml of gentamycin and 10 mM *N*-2-hydroethylpiperazine-*N′*-2-ethanesulphonic acid (HEPES). After 3 days the medium was obtained, filtered and used for culturing retinal explants. In the other type of experiments the optic tectum was co-cultured with the retina. Cultures were performed in the absence of FCS and in the presence of 0.25 mM glucose, as predetermined (Cubillos *et al*., 2002), except when indicated. The retinas were intact (NLR) or with crush of the optic nerve 10 days before plating (LR). Taurine was added in some experiments at concentrations of 0.2, 2, 4, 10, and 20 mM. Sprague-Dawley rats, 5-day-old without lesion of the optic nerve, were also used for measurement of the retinal explant outgrowth in the presence of the goldfish optic tectum or taurine. For their culture, 50% of the medium was minimal essential medium and 50% Leibovitz. The substrate was poly-L-lysine. The neurite length was determined with the program SigmaScanPro (Jandel Scientific) 5 days after plating.

3.2. Effect of Chelating Calcium or Inhibition of Taurine Transport

Chelating agents of extracellular or intracellular calcium, ethylenglycol-bis(β-amino-ethyl ether)-*N,N'*-tetraacetic acid (EGTA, 1 mM) or 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid methyl ester (BAPTA-AM, 0.1 μM) (Lima *et al*., 1993), were added to the culture medium. Guanidoethylsulfonate (GES), an inhibitor of taurine transporter, was added to the medium in some cultures of retinal explants at concentrations of 0.1, 1, 10, and 50 mM, without or with 0.2 mM taurine. GES, 10 mM, was also added to retinal-optic tectum co-cultures.

3.3. Statistical Analysis

Results are expressed as means ± standard error of the mean. Analysis of variance was performed and differences were considered significant when $P < 0.05$.

4. RESULTS

4.1. Effect of Optic Tectum Medium or Retina-optic Tectum Co-culture on Outgrowth From Intact or Post-crush Retinal Explants

The presence of medium from NLOT produced a decrease in outgrowth as compared to basal in the case of LR. The medium from LOT increased outgrowth in a time-dependent manner with the highest values at 5 and 10 days after crush. Outgrowth was significantly higher in LR than in NLR (Table 1).

Table 1. Effect of goldfish optic tectum media on goldfish retinal outgrowth

	NLR	LR
Basal	298 ±23	779 ±57*
NLOT	266 ±24	488 ±29*
LOT-3	465 ±28**	822 ±37*
LOT-5	295 ±25	1071 ±52*,**
LOT-10	220 ±23	904 ±49*,**
LOT-14	319 ±52	516 ±34*,**
LOT-20	0	517 ±26*,**

Length of neurites in μm. NLR, retinas without crush of the optic nerve; LR, retinas with crush of the optic nerve 10 days before plating. NLOT, medium from optic tectum without crush of the optic nerve, LOT, medium from optic tectum with crush of the optic nerve at various days as indicated, and cultured for 3 days prior to collection of the medium. ANOVA: NLR, $F_{(5,99)} = 10.50$, $P < 0.001$; LR $F_{(6,343)} = 20.91$, $P < 0.001$. * $P < 0.05$ *vs.* NLR; ** $P < 0.05$ *vs.* the corresponding basal.

The co-culture of the optic tectum and retina produced an increase in the LR in all conditions, including those corresponding to NLOT (Table 2).

4.2. Effect of Taurine and Optic Tectum on Goldfish Retinal Outgrowth

The addition of 0.2, 2 or 4 mM taurine to LR retinal explants cultured in the presence of medium from optic tectum resulted in no effect of taurine as compared to basal (Table 3). In the experiments of optic tectum and retina co-cultures there was an inhibition of outgrowth by the addition of taurine (data not shown).

Table 2. Effect of goldfish optic tectum in co-culture with goldfish retina on retinal outgrowth

	NLR	LR
Basal	662 ±65	956 ±57*
NLOT	723 ±62	1419 ±5*,**
LOT-1	634 ±52	1527 ±0*,**
LOT-2	569 ±24	1629 ±5*,**
LOT-4	613 ±57	1236 ±6*,**
LOT-7	651 ±41	1351 ±0*,**
LOT-10	619 ±51	1351 ±0*,**

Length of neurites in μm. NLR, retinas without crush of the optic nerve; LR, retinas with crush of the optic nerve 10 days before plating. NLOT, optic tectum without crush of the optic nerve; LOT, optic tectum with crush of the optic nerve at various days as indicated. ANOVA: NLR, $F_{(6,327)} = 0.88$, $P = 0.513$; LR $F_{(6,348)} = 24.29$, $P < 0.001$. * $P < 0.05$ *vs.* NLR; ** $P < 0.05$ *vs.* the corresponding basal.

Table 3. Effect of goldfish optic tectum media and taurine on post-crush goldfish retinal outgrowth

	Without taurine	With taurine (0.2 mM)
Basal	800 ±22	950 ±18**
NLOT	789 ±21	839 ±29
LOT-3	923 ±30**	1039 ±31
LOT-5	1024 ±30**	891 ±28
LOT-10	893 ±27	867 ±28
LOT-14	873 ±29	808 ±29
LOT-20	921 ±35	881 ±37

Length of neurites in μm. LR, retinas with crush of the optic nerve 10 days before plating. NLOT, medium from optic tectum without crush of the optic nerve, LOT, medium from optic tectum with crush of the optic nerve at various days as indicated, and cultured for 3 days prior to collection of the medium. ANOVA: without taurine, $F_{(6,1799)} = 9.56$, $P < 0.001$; with taurine, $F_{(6,1250)} = 29.37$, $P < 0.001$. ** $P < 0.05$ *vs.* the corresponding basal.

4.3. Effect of Calcium Chelators and Inhibition of Taurine Transport

EGTA and BAPTA significantly reduced the outgrowth from the retinas co-cultured with the optic tectum in the absence and presence of taurine. The presence of GES also reduced the effect of taurine alone in a concentration-dependent manner, being effective at 1 and 10 mM. The presence of GES, 10 mM, reduced the trophic effect observed by the co-culture with NLOT or LOT (data not shown).

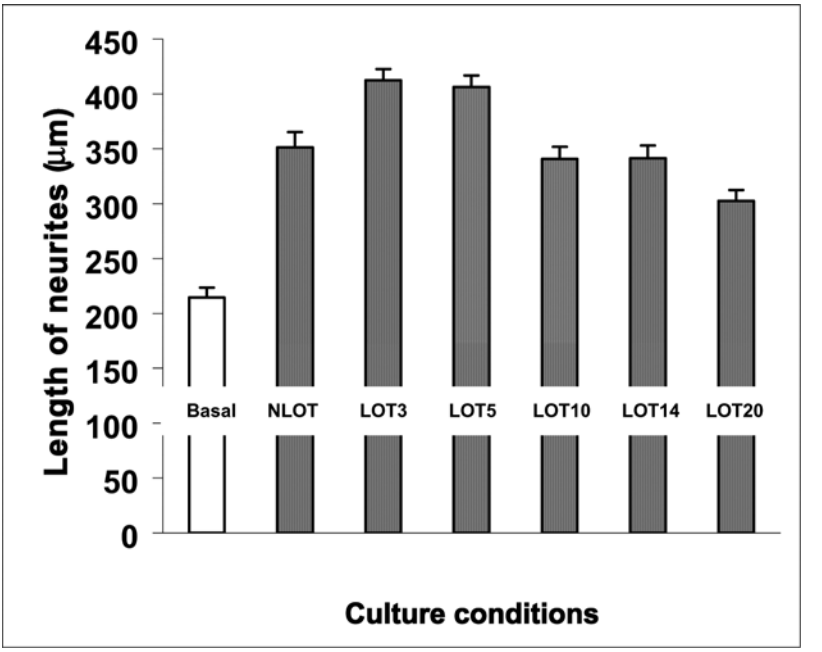

Figure 1. Outgrowth from retinal explants from 5-day-old rats in the presence of medium from the goldfish tectum either without lesion (NLOT) or at various days after the lesion of the optic nerve (LOT). $P < 0.05$ for all conditions *vs.* basal.

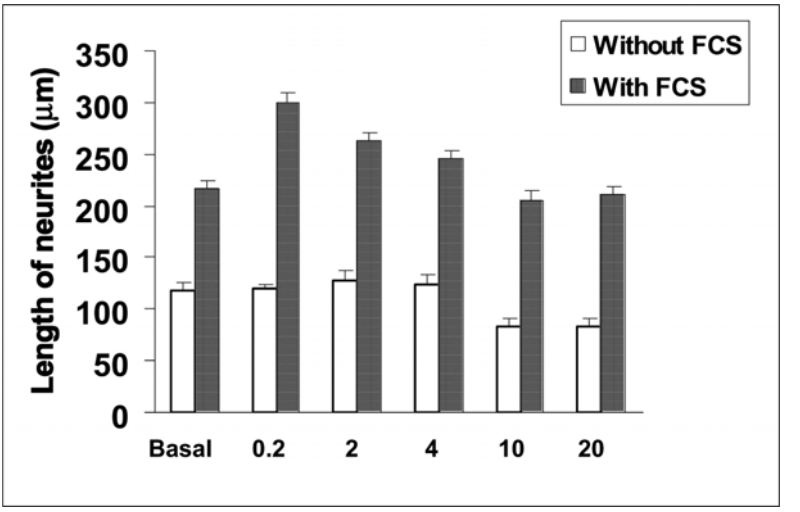

Figure 2. Outgrowth from retinal explants from 5 days old rats in the presence of variable concentrations of taurine (mM) without or with 5% fetal calf serum (FCS). $P < 0.05$ all conditions respecting Basal. $P < 0.05$ 10 and 20 mM without FCS respecting the rest of the conditions; all conditions between without and with FCS; and 0.2, 2 and 4 mM *vs.* basal conditions.

4.4. Effect of Taurine or Goldfish Tectum on Rat Retinal Explant Outgrowth

The addition of medium from the optic tectum of goldfish, either NLOT or at different days after the crush of the optic nerve, increased the length of neurites from 5-day-old retinal explants of the rat (Fig. 1). Taurine added in the presence of FCS produced a trophic effect in these explants, but in the absence of FCS reduced the outgrowth at 10 and 20 mM (Fig. 2). Fig. 3 shows an explant growing in the presence of medium from optic tectum, LOT5.

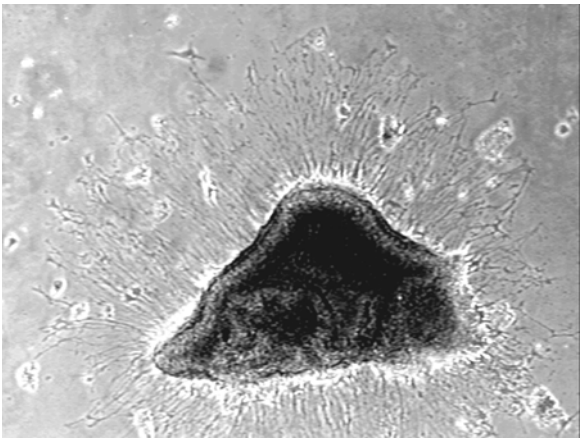

Figure 3. Retinal explant of 5 days old rat in the presence of medium from optic tectum, LOT5, after 5 days in culture in the absence of fetal calf serum. 4 cm = 500 μm.

5. DISCUSSION

The present results indicate that the optic tectum of the goldfish produces factors affecting outgrowth of the retina. In the first place, soluble factors released to the medium when added to retinal explants influence outgrowth. Clear differences are observed between retinal explants without crush or with crush of the optic nerve, expressing much higher emission of neurites in the latter case. Interestingly, the medium of the optic tectum in which optic nerve was crushed around 5 days before plating for obtaining the medium, LOT5, increased outgrowth, but that coming from the optic tectum without lesion of the optic nerve, NLOT, reduced outgrowth. This might be an indication of inhibitory factors from the intact tectum probably directed to the refinement of the innervations impeding further outgrowth. Conditioned media prepared with retinal cells from newborn rats or chick embryos increase survival of rat retinal explants post-optic axotomy (Rehen *et al*., 1993), indicating that trophic factors produced by the retina of distinct species could modulate outgrowth. In addition, a number of molecules have been identified to be related with the establishment of a correct retino-tectal pathway. Among them are factors from the optic nerve of the goldfish, called AF-1 and AF-2, producing nerve regeneration *in vivo* (Schwalb *et al*., 1995). For instance, TAG-1, a

glycosylphosphatidyl inositol-anchored protein, plays a role in the retinotopic organization and regeneration of retino-tectal pathway (Lang *et al.,* 2001). In the goldfish, ganglion cells project to a continuous retino-topic array (Wang *et al*., 2000), in which the retino-tectal topography and its recovery during optic nerve regeneration is modulated by ephrin-A2 in the goldfish, which is expressed in resident neurons and not in new cells (King *et al*., 2004), and has been also implicated in retino-tectal map formation (Rodger *et al*., 2000).

The addition of taurine did not further affect outgrowth from goldfish retinal explants, as an indication of maximum effects of the tectum or inhibition related to bell-shaped time-dependent processes. A relevant difference was observed in co-culture of the optic tectum and retina. The main one is probably related to bidirectional communication between innervating tissue and its target. For instance, NLOT cultured with LR results in outgrowth stimulation. In co-culture, the trophic effect was observed up to 10 days after lesion of the optic nerve of the fish from which the optic tectum was obtained. In this positive condition, taurine reduced the length of neurites, maybe as a result of overexposure to trophic agents. The coordination of time-dependent effects has been demonstrated in a series of processes influenced by trophic agents during the regeneration of the optic nerve of the goldfish (Neuman *et al*., 1983; Lima *et al*., 1988). Critical times for each step process of goldfish optic nerve regeneration have been demonstrated in several experiments.

The effects of the optic tectum on retinal outgrowth, as well as that of taurine, involve calcium, a significant messenger during nerve regeneration (Lima *et al*., 1993). Very interesting are the results with GES, since the inhibition of taurine transport not only decreased the trophic actions of taurine, but also impaired the effect of LOT. It seems that, at least partially, the effects of LOT are related to the entrance of taurine into regenerating cells. Probably some compartments are interacting in the explants, maybe taurine released from Müller or other cells is transported into ganglion cells, which do not contain taurine but express taurine transporter in the goldfish (unpublished results).

Retinal explants from 5-day-old rats, outgrowth in the absence of medium from goldfish optic tectum was stimulated by medium from NLOT and LOT, although a differential time-dependent effect was observed when the goldfish optic tectum and the rat retina were co-cultured. Moreover, taurine increased outgrowth from the rat explants if FCS was also present in the cultures. Rat retinal regeneration after axotomy was demonstrated on laminin (Ford-Holevinski *et al*., 1986), although astrocytes are nonpermissive for regenerating retinal ganglion cell axons (Bahr *et al*., 1995). Interspecies interactions have been reported elsewhere under certain conditions (Ankerhold *et al*., 1998; Li *et al*., 2003).

In summary, the optic tectum of the goldfish produces time-dependent factors after optic nerve crush that modulate outgrowth from the goldfish and rat retinal explants, involving calcium and intracellular taurine mediation.

6. ACKNOWLEDGMENTS

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NEURITIC OUTGROWTH FROM GOLDFISH RETINAL EXPLANTS, INTERACTION OF TAURINE AND ZINC

Sonia Nusetti, Francisco Obregón, and Lucimey Lima*

1. ABSTRACT

Various studies provide evidence for an interaction between taurine and zinc during development, affecting the morphology and function of the retina. The objectives of the present work were to determine taurine and zinc levels in the retina of goldfish during regeneration and to investigate the effect of the intracellular zinc chelator *N,N,N,N*-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) on the trophic role of taurine on outgrowth from post-crush goldfish retinal explants. Taurine was determined by HPLC (nmol/mg protein) and zinc by spectrophotometry ICP (μg/mg protein) at various days post-crushing the optic nerve. The levels of taurine were significantly increased at 72 h and the zinc levels at 24 h. Explants from retinas, 10 days post-crush, were cultured for 5 days in the presence of various concentrations and combinations of TPEN and taurine. TPEN, 1 nM, decreased the outgrowth but simultaneously with taurine (1-8 mM) there was an increase. These results demonstrate that zinc was necessary for normal outgrowth of retinal fibers and that taurine counteracted the chelator effect.

2. INTRODUCTION

Taurine (2-aminoethanesulfonic acid), a β-amino acid, is present at high levels in the retina of many vertebrates (Militante *et al.,* 2002). This amino acid is known to be related to the mediation of multiple functions, such as osmoregulation, modulation of calcium fluxes, neuromodulation, modification of protein phosphorylation, and membrane stabilization, (Lima, 1999). Thus, taurine possesses neuroprotective and

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neurotrophic properties in the central nervous system (CNS) during development and regeneration (Lima *et al.,* 2004). Some taurine functions are similar to those of zinc, an element with relevance in metabolic, genetic, and neurotrofic processes (Grahn *et al.,* 2001). Zinc is highly concentrated in the retina. The physiological functions of zinc have been studied in the retina, where it is believed to interact with taurine, to modify photoreceptor plasma membranes, to modulate synaptic transmission, and to serve as an antioxidant (Grahn *et al.,* 2001). Both taurine and zinc form complexes that seem to be inserted into and to stabilize the plasma membrane and some studies provide evidence for their interaction during development, affecting morphology and function of the rat retina (Gottschall-Pass *et al.,* 1997). The objectives of the present work were to evaluate the possible relationship between taurine and zinc levels in the retina of the goldfish during regeneration of the optic nerve and to investigate how the intracellular zinc chelator *N,N,N',N'*-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) affects the effect of taurine on the outgrowth from post-crush goldfish retinal explants.

3. MATERIALS AND METHODS

Goldfishes (*Carassius auratus*), 3-5 cm in length, were used after a lesion of the optic nerve. They were adapted to darkness for 30 min and anesthetized prior to dissection of the retina. Explants from the retinas obtained after the lesion of the optic nerve (10 days), were plated (10-15 squares per dish) in poly-L-lysine-precoated dishes and cultured for 5 days (Lima *et al.,* 1988). The nutrient medium was Leibovitz (L-15, GIBCO) and combinations of taurine (1-16 mM) and TPEN (0.1-5 nM) (Hyun *et al.,* 2000) were added to the nutrient medium at the moment of plating. TPEN was dissolved in dimethylsulfoxide (DMSO) and the final concentration was 0.025%. Five days after plating, the length (at least five neurites per explant) was determined by using the program Sigma ScanPro (Jandel). Taurine was determined by HPLC with fluorescent detector (nmol/mg protein) employing a modified method (Lima *et al.,* 1998) and zinc by spectrophotometry (ICP-AES) (μg/mg protein) (Lima *et al.,* 2004), at various days post-crushing the optic nerve. Each value is expressed as mean ± standard error of the mean (SEM). Analysis of variance (ANOVA) was used for comparisons. Significance was considered if $P < 0.05$.

4. RESULTS

4.1. Taurine and Zinc Levels in the Retina of Goldfish After Crushing the Optic Nerve

The levels of taurine (Tau) were significantly increased 72 h after the crush of the optic nerve, and returned to normal values at 190 days. Zinc levels significantly increased at 24 h after the lesion and were restored to values similar to control at 3 days (Table 1).

Table 1. Concentration of taurine (Tau) and zinc in the in the retina of goldfish at various days after the crush of the optic nerve

Days after lesion	Tau (nmol/mg protein)	Zinc (μg/mg protein)
Without lesion	55.68 ±1.45	0.0252 ±0.0026
1	71.44 ±1.79	0.0763 ±0.0041*
3	74.74 ±1.44*	0.0406 ±0.0065
5	86.46 ±2.20*	0.0392 ±0.0069
10	88.22 ±1.28*	0.0479 ±0.0039
20	88.60 ±2.39*	0.0326 ±0.0038
90	100.10 ±6.72*	0.0225 ±0.0014
180	60.35 ±3.45	ND

Each value is mean ±SEM, n = 10, * P < 0.05 *vs.* without lesion. ND: not done.

4.2. Effect of *N,N,N',N*-Tetrakis(2-pyridylmethyl)ethylenediamine on Neuritic Outgrowth From Post-Crush Retinal Explants

TPEN at 0.1 or 0.5 nM did not significantly decrease the outgrowth in the absence of taurine. Incubation in the presence of 1 nM TPEN decreased the outgrowth from explants (Table 2).

4.3. Effect of *N,N,N',N*-Tetrakis(2-pyridylmethyl)ethylenediamine in the Trophic Role of Taurine on Neuritic Outgrowth From Post-Crush Retinal Explants

Incubation in the presence of 0.5 nM TPEN blocked the stimulatory effect of 4 mM taurine. The stimulatory effect of taurine on the regeneration of goldfish retinal explants was concentration-dependent up to 4 mM. Taurine, 16 mM, decreased the regeneration of post-crush explants, giving a bell-shaped curve. TPEN, 1 nM, added simultaneously with taurine, reduced the effect of taurine, except at 16 mM (Table 3).

Table 2. Effect of TPEN (nM) on outgrowth from goldfish retinal explants after 5 days in culture in the absence of taurine

Conditions	Length of neurites (μm)
Control	1370.87 ±77.84
DMSO	1299.31 ±56.48
0.1	1206.02 ±46.48
0.5	1185.94 ±64.46
1.0	785.04 ±54.29*
2.5	768.01 ±56.01*
5.0	706.73 ±53.08*

Each value is mean ±SEM, n = 40, * P < 0.05 *vs.* control and DMSO.

Table 3. Effect of various taurine (Tau mM) concentrations on the inhibitory effect of 1 nM TPEN in the length of neurites of post-crush retina explants at 5 days in culture

	Without TPEN	With TPEN
DMSO	1387.01 ±84.15	869.26 ±40.15*
1	1700.01 ±66.15	1331.58 ±64.06**,***
3	1714.04 ±49.35	1466.66 ±83.92**,***
4	2144.02 ±75.35*	1356.22 ±58.12**,***
5	2167.02 ±68.01*	1320.54 ±34.66**,***
8	1381.02 ±44	1254.01 ±40.51**,***
16	984 ±50*,****	965.86 ±54.32*

Each value is mean ±SEM, n = 40, * P < 0.05 *vs.* DMSO, ** P < 0.05 *vs.* 1 nM TPEN, *** P < 0.05 *vs.* the corresponding condition of taurine, **** P < 0.05 *vs.* 1, 3, 4, 5, and 8 mM taurine.

5. DISCUSION

The lesion of the optic nerve produced an increase in the taurine concentration in the post-crush retina of goldfish with a later recover to control values. An increased taurine retinal concentration was previously reported for a short period after crush (Lima *et al.,* 1998) and was suggested that taurine increase could be related to its trophic effects, in the repair of the retinotectal pathway especially useful in the mechanism of formation of new cells (Saransaari and Oja, 2000). The return to basal levels at six months could be related to the restoration of a correct pattern of point-to-point connections between the regenerating optic axons and the optic tectum. These mechanisms require three months or more (Saransaari and Oja, 2000).

The pattern of taurine increase and later decrease during regeneration did not correspond to the pattern seen for zinc which increased 24 h after the lesion, preceding that of taurine and indicating relevant periods for elevated total zinc. It is interesting because the zinc increase during regeneration may be related to the later taurine elevation. Studies with high-resolution electron microscopy indicate that taurine and zinc form complexes seem to be inserted into and to stabilize the plasma membrane (Gottschall-Pass *et al.,* 1997). Among the mechanisms of taurine protective effects in the CNS is its release preventing excitation by glutamate during ischemia (Saransaari and Oja, 2000). Zinc also exerts neuroprotective actions against neuronal death in the brain through the antagonism of glutamate *N*-methyl-*D*-aspartate receptors (NMDA) during ischemia (Chen and Liao, 2003). Moreover, activation of metabolism of retinal cells takes place after the crush of the optic nerve and zinc is necessary for these processes (Huaqing and Amemiya, 2001). The interaction of these molecules during the regenerative period will influence the overall processes and the successful end will be the reestablishment of vision.

Zinc has been shown to be essential for normal embryogenesis and for development of the CNS (Huaqing and Amemiya, 2001). We demonstrate that intracellular zinc is necessary for normal outgrowth of retinal fibers from cultured

explants, since a decrease produced by the chelator TPEN at 1 nM *in vitro* significantly reduced the outgrowth from post-crush explants in the absence of taurine. One of the possible biochemical lesions that has been suggested to underlie the deleterious effects of zinc deficiency on brain functions is an impairment in the formation of the microtubule network (Huaqing and Amemiya, 2001). Zinc specifically binds to tubulin and stimulates brain microtubule assembly *in vitro* (Ahn *et al.,* 1998). Other researches have shown that depletion of intracellular zinc with 1-3 μM TPEN is sufficient to induce macromolecule synthesis and caspase dependent apoptosis of cultural retinal cells and cortical neurons (Ahn *et al.,* 1998).

The intracellular chelator of zinc TPEN, 0.5 nM *in vitro*, blocked the outgrowth stimulated by taurine. It appears that in the presence of taurine there is an elevated demand of zinc, because the stimulatory effect of the amino acid was affected, even if control outgrowth was still evident. These observations indicate that taurine requires an optimal concentration of intracellular zinc for exerting its stimulatory effect. TPEN, 1 nM, decreased the outgrowth from explants in the absence of taurine, but in the presence of this amino acid there was an increase in the outgrowth. These results demonstrate that taurine stimulation of outgrowth is evident even in conditions of discrete zinc reduction. It might be that when TPEN lowered intracellular zinc to a critical level, some of the routes of zinc intake or release were activated in the cell and taurine could still increase the outgrowth from the explants.

In summary, the retinal levels of taurine and zinc are elevated after the crush of the optic nerve of the goldfish with a different time-dependent pattern, but probably related to each other.

6. ACKNOWLEDGMENT

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Part 6.
Taurine Derivatives

TAURINE, TAURINE ANALOGUES, AND TAURINE FUNCTIONS: OVERVIEW

L. Bianchi, M. A. Colivicchi, C. Ballini, M. Fattori, C. Venturi, M. G. Giovannini, J. Healy, K. F. Tipton, and L. Della Corte*

1. INTRODUCTION

In view of the high (mM) concentrations of taurine found in most mammalian tissues, it is not surprising that it has a multiplicity of functions. Indeed, it may be an oversimplification to search for a single action that might account for its behaviour in any one system. It is believed that taurine has an enantiostatic role, preserving constant, or almost constant, tissue function in response to external perturbations (see Huxtable, 1992, 1999; Khan *et al.,* 2000). That would account for its apparent cytoprotective actions in a number of systems, although the mechanisms involved may differ, depending on the nature and severity of the toxic perturbation. Indeed, a wide diversity of functions has been ascribed to taurine, which may contribute to its beneficial actions. These include modulation of intracellular calcium homeostasis, membrane stabilisation, osmoregulation, reactive-radical scavenging, oxidative stress, neuroprotection and neurotransmission (see Huxtable 1992, 2000; Della Corte *et al.,* 2002, for reviews).

The use of taurine analogues that possess only some of the properties of taurine has aided our understanding of those properties that may predominate under different stress conditions (see Marangolo *et al.,* 1997; Palmi *et al.,* 1999). Such an approach has, for example, been used to show that the protection by taurine against the toxicity of 1-methyl-4-phenypyridinium (MPP^+) results from its action as a GABA receptor agonist (O'Byrne *et al.,* 2000). An extension of this approach would be to take consideration of the fact that some of the proposed functions of taurine require it to enter the cell and others do not. For example, the action of taurine on GABA receptors (see e.g., Kontro *et al.,* 1990; O'Byrne *et al.,* 2000) is extracellular, whereas its actions as a modulator of mitochondrial calcium-ion transport (Palmi *et al.,* 1999, 2000) require that it enters the cell. A scheme illustrating the possible use of different taurine analogues for making distinctions between intracellular and extracellular

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Taurine 6
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activities is shown in Fig. 1. However, it should be noted that most of the data concerning cellular uptake refer to transport through the specific cellular GABA/taurine transporters (see Marangolo *et al.,* 1997) and the existence of alternative processes cannot be excluded.

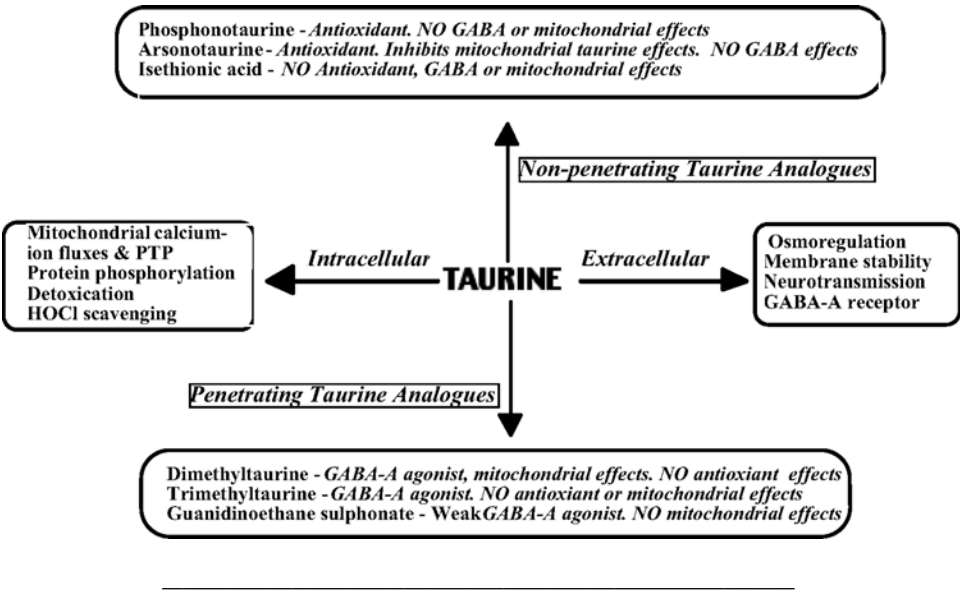

Figure. 1. Some proposed functions of taurine, illustrating how different taurine analogues may help to determine the relative importance of the different functions in responses to specific types of stress.

An alternative approach to investigating the multifaceted behaviour of taurine is to consider the responses to different levels of stress. For example, the release of taurine that accompanies neuronal stimulation has been ascribed to its osmolarity-regulating function, in response to neurotransmittter release and reuptake (see Huxtable, 1992). Some studies to investigate whether taurine release may simply represent a response to the stressful conditions induced by the release of the neurotransmitter glutamate are provided in this account. Two types of stimulus were compared. The "physiological" stimulus was exposure of male rats to a novel stimulus (open field) and its habituation, while monitoring amino acid release from the ventral hippocampus. "Chemical" stimulation was performed by local application of the excitatory amino acid receptor agonist kainate (KA), while monitoring release from the striatum (Str) or globus pallidus (GP).

2. MATERIALS AND METHODS

All the experiments involving laboratory animals were performed according to the Italian Guidelines for Animal Care (D.L. 116/92), which were also in accordance with European Communities Council Directives (86/609/ECC). All efforts were made to

minimise animal suffering. Microdialysis was performed in freely-moving male Wistar rats (175-200 g; Harlan-Nossan, Milan, Italy) as previously described (Bianchi *et al.,* 1998).

In the open-field study the microdialysis probe was positioned in the ventral hippocampus using the following stereotaxic co-ordinates derived from Paxinos and Watson (1982), AP = -5.5, L = -4.8V = -7.8, relative to bregma and dural surface. The open-field arena is illustrated in Fig. 2. Rats were placed in the arena for two 30 min periods, with a 60 min interval between (see Bianchi *et al.,* 2003 for further details) Motor activity was measured every 10 min.

Dual-probe microdialysis, performed as previously described (Bianchi *et al.,* 1998; Galeffi *et al.,* 2003), was used to study the effects of chemically evoked release. One electrode was located in the striatum (Stereotaxic coordinates relative to Bregma: AP 0.7, L 3.2, DV 5.5 mm) and the other in the globus pallidus (AP -1.0, L 3.2, DV 7.5 mm).

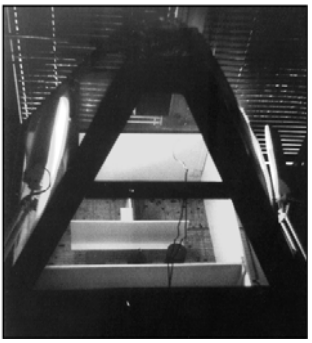

Figure 2. The open-field apparatus used for the studies on exploratory behaviour. This comprised a white-coloured polyvinyl chloride box (78 X 69 X 36 cm) with a grid floor. The arena was illuminated by two 75-W lamps suspended 50 cm above the box. A microwave sensor was located 70 cm above the arena to measure motor activity.

The extracellular amino acid concentrations were determined in the microdialysis samples by HPLC separation followed by fluorimetric detection of their *o*-phthalaldehyde (OPA) derivatives, as described by Bianchi *et al.* (1999). Acetylcholine (ACh) was determined by HPLC with a post-column enzyme reactor and electrochemical detection (Damsma *et al.,* 1987; Giovannini *et al.,* 1994).

The levels of amino acid in the perfusate fractions were expressed as fmol/µl of perfusate (nM). For statistical analysis net output values were used, i.e. the differences between stimulated and basal release.

3. RESULTS AND DISCUSSION

As we have previously shown (Bianchi *et al.,* 2003), there was intense motor activity when the rats were first placed in the arena (249 ± 49 s, n=5), which was accompanied by a significant release of ACh (264% of basal values), aspartate (315%) and, to a lesser

extent, glutamate (181%). This release was maximal after about 10 min and had returned to the basal levels after about 40 min. The results obtained in these open-field experiments are summarised in Table 1. As can be seen the release of these neurotransmitters was not accompanied by any change in extracellular taurine levels.

Table 1. Extracellular levels of acetylcholine, aspartate, glutamate and taurine in the ventral hippocampus at rest (basal), during the first 30 min after being placed in the open-field arena (exploratory)

	Acetylcholine	Aspartate	Glutamate	Taurine
Basal	114 ± 18	3570 ± 840	7020 ± 2370	4020 ± 770
Exploratory	228 ± 33	7320 ± 2100	9060 ± 2580	4132 ± 823
Exploratory - Basal	$P < 0.01$	$P < 0.05$	$P < 0.01$	n.s.

Values are expressed as mean AUC ± SEM (nmol/10 min); n= 8-9.

Table 2. Release of glutamate aspartate and taurine evoked by kainate (100 μM) in the presence and absence of TTX (3 μM) and DNQX (10 μM)

	Striatum (KA/TTX/DNQX in striatum)			
	Basal	KA	KA + TTX	KA + DNQX
Glutamate	216 ± 41 100 ± 19	250 ± 25*	103 ± 16	99 ± 18
Aspartate	232 ± 26 100 ± 12	219 ± 32*	111 ± 20	107 ± 22
Taurine	976 ± 113 100 ± 12	588 ± 69*	530 ± 118*	506 ± 75*
	Globus pallidus (KA/TTX/DNQX in striatum)			
Glutamate	236 ± 39 100 ± 17	188 ± 28*	109 ± 14	97 ± 20
Aspartate	251 ± 23 100 ± 11	213 ± 35*	91 ± 23	113 ± 27
Taurine	561 ± 46 100 ± 8	152 ± 14*	95 ± 13	103 ± 13
	Globus pallidus (KA/TTX/DNQX in globus pallidus)			
Glutamate	403 ± 95 100 ± 24	186 ± 52*	108 ± 12	110 ± 11
Aspartate	332 ± 96 100 ± 29	161 ± 41*	122 ± 12	105 ± 13
Taurine	443 ± 66 100 ± 19	164 ± 34*	102 ± 15	106 ± 10

Values of mean AUC ± SEM (nM/20 min fraction, n= 8-9) are expressed as percentage of basal values *= $P < 0.05$ against basal value (ANOVA followed by *post hoc* LSD test).

These results indicate that taurine release is not a necessary accompaniment to neurotransmitter release and, perhaps, suggest that its well-documented release in response to K^+- or ligand-evoked neurotransmitter release is a reflection of the severity of the conditions used in such studies. Thus, the enantiostatic function of taurine in regulating osmolarity may appear predominant under conditions of extreme stress.

Similarly, the elevated levels of extracellular taurine that are found in human glioma tumour tissue and which correlate with the degree of cell proliferation (Bianchi *et al*., 2004) may reflect an association between increased taurine and oedema, since cell proliferation leads to peritumoural oedema, as a result of deficient perfusion arising from elevation of regional tissue pressure.

The results for the evoked release of taurine in the GP, summarised in Table 2, would be consistent with it being a stress response. Local stimulation of the Str with 100 μM KA induced a significant release of aspartate and glutamate (200%), which was accompanied by a massive taurine release (600%). However, this taurine release was not dependent on action potential propagation, since it was unaffected by the presence of tetrodotoxin (TTX, 3 μM). Neither was it sensitive to inhibition by 6,7-dinitroquinoxaline-2,3-dione (DNQX), which is an antagonist of the non-NMDA glutamate receptors (see Bianchi *et al*., 1994, 1998). Thus, this taurine release does not involve the activation of non-NMDA receptors and may be extraneuronal.

In contrast, when the release was measured distally in the GP or when the KA stimulation was applied locally to the GP, the release of aspartate, glutamate and taurine was similar in magnitude (see Table 2). Furthermore it was inhibited by both TTX and DNQX, indicating it to be dependent on the propagation of action potentials and the activation of non-NMDA receptors.

Thus, the data presented here show that taurine release does not necessarily accompany glutamate (or aspartate) release and that its release in response to chemical stimulation may, in some cases, reflect a response to the severity of the stress accompanying chemically evoked release. Such a stress response would be in accord with the observation that the release of taurine in Str following kainate challenge did not depend on depolarisation or kainate-receptor activation. However, the release evoked in the GP may indicate a more specific role for taurine release that is not simply a stress response. Clearly, it is necessary to distinguish between these two types of release when attempting to understand the functions of taurine.

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TAURINE ANALOGUES AND TAURINE TRANSPORT: THERAPEUTIC ADVANTAGES

R. C. Gupta*

1. INTRODUCTION

Taurine, 2-aminoethanesulfonic acid, is a β-sulfur amino acid. Though it was first isolated about 170 years ago from ox bile, in fact it was discovered about 40 years ago with the publication of an excellent review (Jacobsen and Smith, 1968), which opened new ways for investigation of this old molecule. Taurine has disjunctive distribution in the biosphere, but more in the animal kingdom than in cell-walled structures. In animals, its presence can be noticed from unicellular organisms to highly developed mammals. In general, it is present in high concentrations, which also provides the logic for its involvement in a variety of functions from osmoregulation to cardioprotection and from hypertension to the neurotransmitter role. All these actions make taurine a poly-functional molecule, involving its role in maintenance of various live-forming processes. This wide-spread involvement of taurine seems to be due to its unique physicochemical nature, which is provided by special features of the β-methylene chain of two molecules with one end occupied by a primary amino group and the other by a sulfonic group; not the carboxylic group which is a part of the majority of neuroactive amino acids. These differences also make a sharp difference in the common physical constants and affect physical properties, like pH, diffusion, solubility, ionization, and isoelectric points, thus making taurine strongly zwitterionic in nature with high water solubility and low lipophility, hence slowing diffusion across membranes. This entire phenomenon affects taurine transport, which is also influenced by the membrane lipid composition.

The intracellular levels of taurine in mammalian tissues are determined by the equilibrium between biosynthesis, intracellular amounts, and excretion rates. The capacity to synthesize taurine varies widely among animal species and is determined by an oxidative process, in which methionine and cysteine are finally metabolized to taurine, involving various enzymes. However, many mammals have lost the ability to synthesize taurine and relay on nutritional sources only. For instance, cats are totally dependent on

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Taurine 6
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dietary sources. Dietary taurine, when available, gradually decreases the synthesizing ability: less taurine is produced, but however the excess of taurine is eliminated through urine.

The process of intake from the intestine, where taurine recovered from dietary sources is accumulated, supports exchange of taurine between organs, tissues and cells. Also excretion from the kidney takes place and all these actions depend on transport through cell membranes. The presence of taurine in considerably high concentrations in the central nervous system (CNS), retinal membranes, heart, skeletal muscles and platelets, much higher than in blood plasma, provides strong evidence of active transport processes between intercellular and extracellular spaces. In search of further identifying the transport mechanisms, two transport systems for taurine have been identified. They differ only in their affinity for taurine. In addition to this, a third non-saturable transport system, attributable to diffusion across the membranes, also exists which may also influence transport.

For transport of taurine, cellular release of taurine is an important event and there are three different types of cellular release. Each may be involved in a variety of phenomena. The release of taurine depends on the permeability of membranes to taurine, but also varies according to the lipid composition and other factors. It is believed that for functional regularity a continuous pool with a sufficient amount of taurine is essential. If not, functional or structural abnormalities can be detected in important organs like heart, retina, and brain. Hence a good taurine pool is a must, but the ionic nature of taurine and its steric and electronic similarity to membrane phospholipids and strong acidic character makes it zwitterionic at physiological pH. This results in impermeability of taurine to bilayer membranes. However, its analogues like β-alanine or GABA can diffuse through such membranes. The lack of liposolubility of taurine allows cells to maintain steep concentration gradients and contribute towards limited availability of taurine for the hour of need. This situation easily explains the only partial clinical success when taurine is given orally in epilepsy or some other disease conditions but a good response when administered intraperitoneally. Otherwise, extremely high doses with quite long duration are required to achieve any clinical efficacy. Hence, to overcome the majority of such problems, taurine analogues with improved liposolubility and enhanced absorption are needed, facilitating the release and transport of taurine across the blood-brain barrier. Such analogues probably release their taurine moiety at the site of request hence increasing the therapeutic potential. (Della Corte *et al*., 2002) In short, taurine analogues or pro-drugs of taurine with the basic taurine skeleton intact may provide a good source of taurine, with easy transport to the target site for enhanced biological actions.

1.1 Modification of Taurine Contents

Taurine contents can be modified effectively by the use of agonists and antagonists or through dietary alterations. Taurine still lacks a potent and effective antagonist. However, 6-aminomethyl-5-methyl(4H)-1,2,4-benzothiadiazine-1,1-dioxide (TAG) is one well-known synthetic antagonist (Yarbrough *et al*., 1981). In the absence of a potent antagonist most of taurine depletion studies have been carried out with β-alanine or guanidinoethanesulfonate (GES) (Huxtable *et al*., 1979, Mori *et al*., 2000). A solution of 1% GES in drinking water causes 80% taurine depletion within 6-8 weeks of treatment in all major tissues. The use of β-alanine gave almost the same depletion rate and turnout.

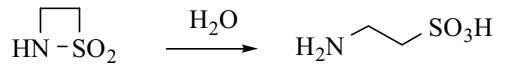
1.2. Why Analogues?

Amino acids are poorly absorbed in the gastrointestinal tract and the ratio between the doses administered orally and the corresponding levels attained in the CNS are very unfavorable. This situation arises from the fact that the transfer of amino acids from the gastrointestinal tract into the CNS is limited by an active transport mechanism that has a finite capacity. Passive diffusion of amino acids is negligible due to their polar character and highly ionized state. Poor absorption is accompanied by high losses through metabolic degradation, which occurs in both the gut and liver. These losses constitute the well-known 'first-pass effects'.

Taurine, being an amino acid, observes all these conditions. However, it has several specific extra problems: unfavorable pharmacokinetics, very strong hydrophilic nature, lipophobic character, and fast rate of extraction through urine. All this apparently affects its significance as a therapeutic agent. For example, taurine is a potent anticonvulsant in animal epilepsy models and when administered intracerebroventricularly it effectively prevents seizures in these animals. However, it has failed to show such effects when given orally or administered peritoneally, and an extremely high dose, typically greater than 3 g per day, is required to achieve any clinical efficacy.

The synthesis of suitable pro-drugs of taurine may afford a way for improving passive diffusion and circumventing some of the first-pass effects. Hence, a pro-drug of taurine may have following features:

- Minimum chemical features required for biological activity must be identified and preserved.
- When increasing the lipophilic character, it should also be taken care of water solubility. The use of organic solvents is not always beneficial as precipitation at later stages might occur.
- It is highly desirable that the pro-drug synthesized should diffuse quickly across the membrane to undergo then transformation to the target molecule, i.e. taurine, in the cell. From this point of view, cyclic analogues of taurine such as β-sultam will be of special value as they are slowly hydrolyzed in water to yield taurine (Fig. 1).



β-Sultam **Taurine**

Figure 1.

- A two-carbon atom chain is a prerequisite for activity preferably in the form of methylenic groups.
- A sulfonic group is definitely required. It can also be in the form of sulfone or sulfonamide. A further reduced sulfonic moiety may not be effective.
- An amino group, preferably a primary one, is needed for optimum activity. - However, lipophilization of taurine can be achieved by converting the primary amino group into more lipophilic and less ionized secondary or tertiary amines.

- For superior activity both the amino (or substituted amino) and the sulfonic (or substituted sulfonic) groups have to be directly attached to the methylenic chain.
- In general, substitution of the methylenic chain, leads to inhibition of the desired effects.
- Pseudo-cyclic, as well as cyclic analogues, of taurine may exhibit taurine-like activity under similar circumstances.
- The preferable substitutions to be attached to the amino or sulfonic moieties should be small alkyl groups or aryl groups but bulky alkyl groups diminish activity.

It seems from the above considerations, that the analogues may have as essential features a skeleton structure as shown below (Fig. 2). It will be a good practice to preserve it while developing new taurine derivatives or analogues.

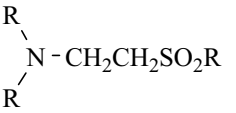


Figure 2.

1.3. Taurine Transport

In general, transport systems for taurine are specific for β-amino acids and require energy and temperature. In addition to the temperature and energy requirement, transport or binding to the transport site is also Na^+-dependent. The structure-activity characteristics of the taurine transporting system are very similar from unicellular organisms to highly complex mammals. Two saturable transport systems for taurine have been identified, differing only in their affinity for taurine. Another third non-saturable component, attributable to diffusion across the membranes, is also acknowledged. Several workers using a wide range of taurine concentrations have found these two-component systems (Huxtable, 1989; Yan *et al*., 1999).

As active transport of taurine is Na^+-dependent, the ratio of Na^+ ions to the taurine molecules transported varies from 3:1 to 1:1. For example, in hypothalamic synaptosome transport one Na^+ ion per taurine molecule is needed, in Ehrlich ascites cells the ratio is 2:1, and in brain cells 3 Na^+ are needed for transport of taurine. It is believed that Na^+ increases the affinity of transport, but in some cases Na^+ increases the V_{max} of transport. Taurine transport is also influenced by other ions. For example, placental brush border membrane vesicles transport is Cl^--dependent.

Many other ions like SCN^-, NO_{3-} and SO_4^{2-} have less influence. K^+ stimulates taurine efflux from primary astrocyte cultures but this process, in addition to Na^+, also requires Ca^{2+} ions. Many taurine analogues, like hypotaurine, β-alanine, and GES, are potential inhibitors of taurine transport but the actual mechanism is yet not fully explained. The membrane lipid composition also affects taurine transport. Supplementation of Y79 cells with arachidonic acid facilitated taurine uptake by lowering the K_m of the high-affinity transport system. It is also important to note that ionic and structural similarities between taurine and the zwitterionic head groups of neutral phospholipids, phosphatidylcholine and phosphatidylethanolamine also play important roles in binding and transport,

suggesting that dipole-dipole interactions of taurine with the neutral phospholipids increase the affinity of calcium to the high-affinity binding site of acidic phospholipids. Taurine transport in important organs such as brain, heart, liver, lungs, and many others have been characterized in literature. Such interactions are believed to be the cause of several biological activities produced by taurine.

2. TAURINE ANALOGUES AND BIOLOGICAL ACTIONS

2.1. CNS Actions

Taurine is the most abundant amino acid in the brain and found in all cell types in the CNS. High concentrations of taurine occur in the developing brain. The activity of taurine is probably mediated via its binding to GABA or glycine receptors. It is believed that taurine acts at a unique population of receptor sites. Taurine modulates the release of GABA but their biochemical actions are different. Taurine analogues along with taurine itself have been studied. While some analogues act as taurine does, others behave differently. Such behavior may be due to a unique receptor having two sites. One site is negatively charged and the other positively, interacting with both the negative and positive functional groups of taurine. The antiepileptic action is the most prominent activity of taurine. A number of taurine analogues have been synthesized and evaluated for anticonvulsant actions (Sgaragli *et al*., 1994). Antiepileptic activity has also been exerted by homotauryl-homotaurine, carbobenzoxyhomotauryl-homotaurine, and γ-aminobutyltaurine. These compounds demonstrated similar activities to those of the unsubstituted precursors. The antiepileptic activity occurs via interacting with the GABA binding site in the brain. However, carbobenzoxyhomotaurine, homotaurine, and γ-glytamyltaurine have not exhibited any affinity to the GABA binding site.

In search of potent anticonvulsants, a series of 2-acylamidoethanesulphonamides have been synthesized (Lindén *et al*., 1983; Oja *et al*., 1983) (MY series in Table 1). The core structure for taurine-like activity is preserved and the compounds are less polar, less ionized and more lipophilic than taurine, thus exhibiting increased lipid solubility. The anticonvulsant activity was evaluated in the subcutaneous pentylentetrazole seizure threshold (PST) test. Phthalimidoethanesulfonamide derivatives of taurine were also prepared and studied (Table 1). The activity was higher with derivatives containing less alkyl groups. Thus the methyl, dimethyl, and isopropyl derivatives, and even the unsubstituted amide, were more active.

The same group extended further their study with phthalimido derivatives and found that 2-phthalimidoethanesulfonamide (MY-103) (see Table 1) is a very effective in the maximal electroshock seizure and PST tests in mice. Several of the active compounds were also studied for taurine uptake in the mouse brain. Only taltrimide (MY-117, 2-phthalimidoethanesulfon-N-isopropylamide) inhibited the uptake. Taltrimide and MY-103 were also tested against genetically and experimentally produced seizures. Taltrimide was found to raise the threshold for audiogenic seizures and to decrease seizure severity (Nakagawa and Huxtable, 1985).

To introduce taltrimide for use in epileptic patients, clinical trials, including anti-epileptic effects and effects on EEG have been performed. Taltrimide penetrated well through the blood-brain barrier and its main metabolite phthalimidoethanesulfonamide was found in the cerebrospinal fluid. The two active derivatives MY-103 and MY-117

were further evaluated for affording cerebral osmoprotection during chronic hypernatremic dehydration (Trachtman *et al*., 1988).

Table 1. Analogues of taurine

R_1-CH_2CH_2-SO_2-R_2

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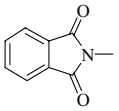
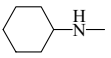
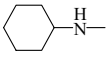
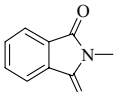
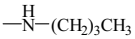
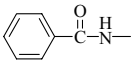
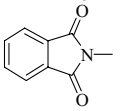
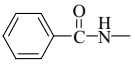
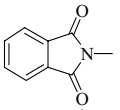
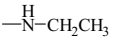
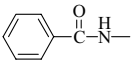
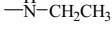
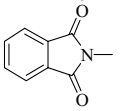
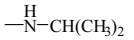
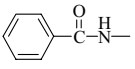
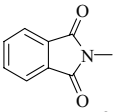
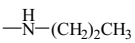
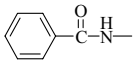
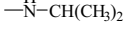
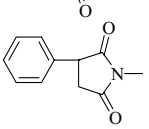
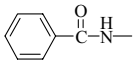
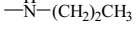
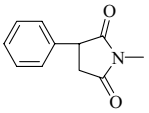
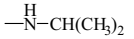
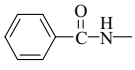
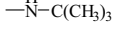
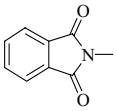
MY 100			MY 111		
MY 101			MY 112		
MY 109			MY 115		
MY 120			MY 116		
MY 121			MY 117		
MY 122			MY 118		
MY 119			MY 135		
MY 123			MY 136		
MY 102					
MY 103					

The promising anticonvulsant activity shown by several taurine analogues and the development of taltrimide will have a long lasting effect on the search of even more potent taurine derivatives and analogues.

2.2. Antiaggressive Action

Aggressive display is an attempt to endanger the integrity of the opponent. Taurine shows antiaggressive effects and several taurine analogues with a free amino group or with a phthalimido moiety have been synthesized and studied for aggressive behavior (Mandel *et al*., 1985). The lipophilic derivative 2-phthalimidoethanesulfon-N-isopropyl-amide (MY-117) and taurinamide (MY-107), were more effective than taurine itself at a concentration of 1.6 mmol/kg (per os). Several sulfonamide derivatives of taurine (e.g. with heterocyclic amines) were similarly tested by administration into the olfactory bulb i.p. or per os. All derivatives were more potent than taurine when administered i.p. NCS 702 (see Fig. 3) was more potent than taurine when given into the olfactory bulb, but NCS 704, and NCS 705 were the most effective compounds. It is believed that similarly to GABA-mimetic drugs, taurine analogues can also inhibit muricidal activity.

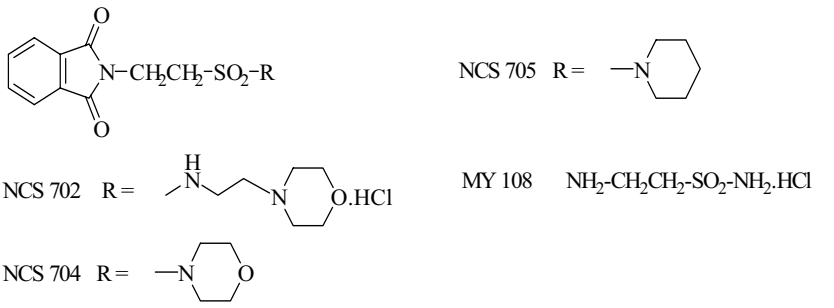


Figure 3.

2.3. Temperature-Lowering Effect

Taurine is involved in thermoregulation via interaction with the central serotonergic system. A number of taurine analogues along with taurine were studied for their effect on body temperature (Fig. 4**)** (Frosini *et al*., 2000). Two sulfonic derivatives were found to affect body temperature. However, phosphonic derivatives abolished the effect and act as antagonists. Replacement of the amino group by a guanidinium group further potentiates the effect of taurine on body temperature. It is believed that the thermoregulatory effect of taurine and its derivatives results from (i) an increased heat loss due to the reduced peripheral vasomotor tone, and (ii) from a decrease in core temperature. Some further taurine analogues were also tested which included *cis*- and *trans*-isomers of 2-aminocyclohexanesulfonic acid (Fig. 4) (Cordero *et al*., 2002). *Cis*-2-aminocyclohexane sulfonic acid was able to induce hypothermia. It was not mediated by the GABA receptor but was due to interaction with the putative taurine receptor in the brain. The *trans*-isomer showed no activity, which might be due to the altered distance between the amino and sulfonic groups in the derivative.

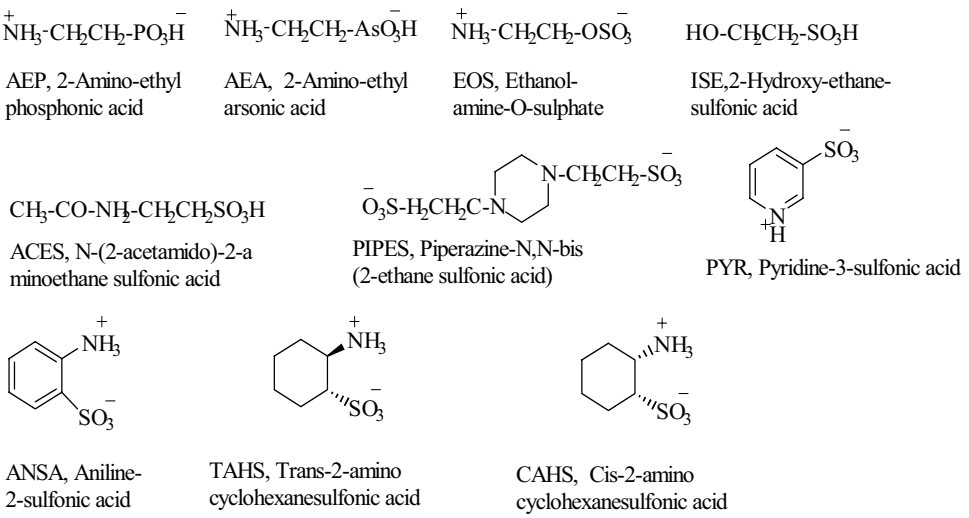


Figure 4.

2.4. Vision

Taurine pool is the most abundant among all amino acids in the retina (10-50 mM). It is involved in the protection of retinal structures and its functions. Taurine is a must for normal vision and its deficiency leads to retinal degeneration and blindness. Such phenomena were observed in cats, rats, and monkeys, while in humans it is still a subject of debate. Taurine also inhibits phosphorylation of specific retinal proteins and provides protection to the rod outer segments (ROS) in frogs exposed to ferrous sulfate. In order to achieve faster and longer protection, several cyclic taurine analogous (with restricted molecular movements) have been synthesized and studied (Liebowitz *et al*., 1986). The sulfone derivatives, AEMS, TMS, and M-TMS (Fig. 5) have been found to be more potent than taurine in stimulating ATP-dependent calcium uptake in the retina. *Trans*-2-aminocyclohexanesulfonic (TAHS) and *trans*-2-aminocyclopentanesulfonic (TAPS) acids inhibited ATP dependent calcium uptake in the micromolar range. On the contrary, *cis*-2-aminocyclohexanesulfonic acid (CAHS), similarly to taurine, stimulated calcium uptake in the millimolar range. TAHS and TAPS are the most potent inhibitors of ATP-dependent-calcium uptake in the rat retinal preparations yet recorded. AEMS, ATS, and APS (Fig. 5) were found to be the most potent stimulators of ATP-dependent Ca^{2+} uptake. AEMS, AST, APS, and TMS inhibited ATP-independent Ca^{2+} uptake and ATS inhibited ATP-dependent Ca^{2+} uptake even at high Ca^{2+} concentrations. A similar effect of these sulfone derivatives was also observed on incorporation of phosphate into retinal proteins. Thus along with taurine, AEMS, ATS, APS, and TMS were equipotent inhibitors of phosphate incorporation (Lombardini *et al*., 1990, 1997).

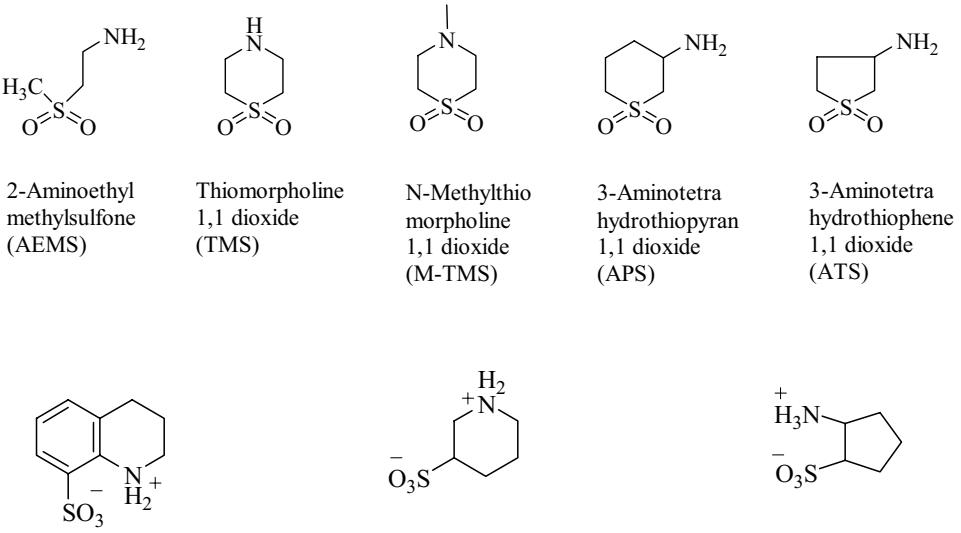


Figure 5. Sulfone analogues and derivatives.

More analogues were evaluated as stimulators and inhibitors of ATP-dependent Ca^{2+} uptake in the rat retina. Among them THQS, PSA and TAPS (Fig. 5**)** have been reported to inhibit ATP-dependent Ca^{2+} uptake and are non-competitive with taurine. THQS with its aromatic structure was found to be a less potent inhibitor than TAPS and is non-competitive with taurine.

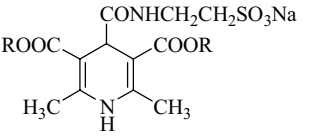
2.5. Cardioprotective Actions

Taurine contents are high in the mammalian heart, ranging from 5 μmol g^{-1} to 40 μmol g^{-1} wet weight. The lack of synthetic ability in cardiac tissues proves that taurine originates from transport processes. Although limited taurine synthesis in the heart has been reported, most of taurine is accumulated in cardiac tissue by uptake. The uptake from blood is Na^{+}-dependent and two different uptake systems have been suggested. Taurine binding to the heart is also temperature-dependent. The metabolic rate of taurine in the heart is very slow. Increases or decreases in its content can be recorded using agonists or antagonists. There is a direct connection between the taurine deficiency and abnormal cardiovascular system development and a possible link to cardiomyopathy. The cardioprotective action of taurine results from its involvement in the in site mediation of cardiac functions such as inotropic, antiarrhythmic, osmoregulation, hypotensive, and related actions.

Taurine has dual inotropic properties. At high Ca^{2+} concentrations taurine produces negative inotropic effects while at low Ca^{2+} concentrations it exhibits positive inotropic

actions. Taurine also shows antihypertensive properties, lowering the blood pressure and further easing the complexicity of hypertension. It is believed that taurine interacts mostly with cations rather than with anions and more specifically with divalent calcium ions and takes part in the stabilization of membranes.

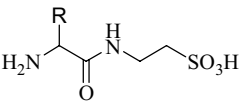
A series of 2,3-dimethyldihydroisonicotinoylaminoethanesulfonate (tauropirone) derivatives have been also synthesized and studied as inhibitors of platelet aggregation (Poikans *et al*., 1994) (Fig. 6). The ethoxy derivative was found to be the most powerful antiaggregant. It markedly lowered platelet aggregation at a 30-fold lower concentration than taurine. This compound showed also lipid peroxidation activity in human blood plasma, suggesting antioxidative properties. In addition, it increased the superoxide dismutase activity in plasma by 17.5% (in comparison to 2% by taurine).



R = -CH_3, -C_2H_5, -C_3H_8

Tauropirones

(2,3-dimethyldihydroisonicotinic acid derivatives)

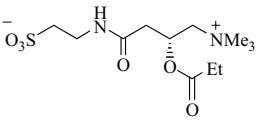


Taurine Peptides

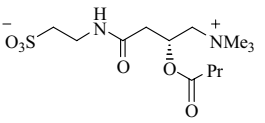
Figure 6.

In search of inhibitors of platelet aggregation, a series of taurine peptides (Fig. 6**)** has also been synthesized (Koyama *et al*., 2003). Most of the peptides (sweet in taste) inhibit platelet aggregation to some extent.

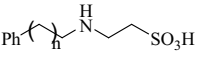
Several taurinamide derivatives (Molaparast-Saless *et al*., 1988), PCTA (propionyl-carnitinetaurinamide) and BCTA (butyrylcarnitinetaurinamide) (Fig. 7**)** exhibited cardio-protective activity at a lower concentration than taurine.



PCTA



BCTA



TAU-Series

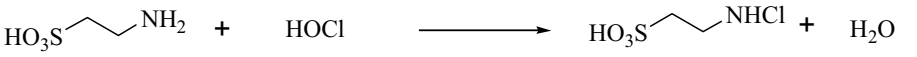
Figure 7.

Taurine is involved in protein phosphorylation in the heart. Analogues like piperidinesulfonic acid and 1,2,3,4-tetrahydroquinoline-8-sulfonic acid (Lombardini, 1994) (Fig. 5) possess similar inhibitory activity when tested *in vitro* using a subcellular mitochondrial fraction of the rat heart. Other two analogues, 2-aminoethylmethylsulfone

(AEMS) with maximal conformational flexibility around the amino and sulfone moieties and (±)3-aminotetrahydrothiopyran-1,1-dioxide (APS), in which the sulfone moiety is a part of a six member ring, were found to be more potent inhibitors of phosphorylation than taurine. Lately, efforts were made to find more potent analogues, which resulted in formation of a phenylalkyl derivative of taurine (TAU-5) (Sapronov *et al*., 2000) (Fig. 7**).** Intravenous infusion of TAU-5 during electrostimulation normalizes abnormal hemodynamic properties such as increased peripheral resistance, decreased cardiac output, and aortic pressure. All the above analogues provide hope to the vast number of people suffering from cardiovascular diseases. An accelerated rate of search is needed in this field.

2.6. Antioxidation and Related Actions

Several free radicals are produced in the body as byproducts of normal metabolism and also upon exposure to radiation and various environmental pollutants. They are highly reactive, causing damage to cellular components and leading to a variety of diseases. These free radicals are also known as reactive oxygen species (ROS) and include super oxide (O^{2-}), hydroxyl radical (OH^{-}) and hydrogen peroxide (H_2O_2). They contribute towards cytotoxicity, morphological and metabolic changes, changes in the CNS, and increased muscle proteolysis. Antioxidants play an important role in health maintenance. Taurine has been recognized as an antioxidant long time ago, scavenging both ROS and nitrogeneous radicals. Such radicals also combine with halogen ions (e.g. chloride, bromide) in a reaction catalyzed by myeloperoxidase, producing hypochlorous or hypobromous acid (Evans and Hilliwell 2001).



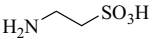
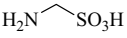
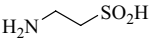
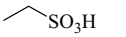
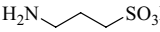
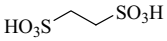
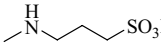
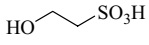
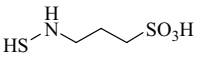
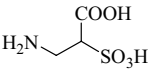
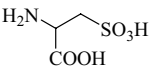
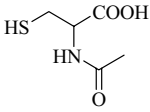
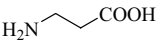
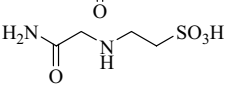
Taurine Hypochlorous acid N-Chlorotaurine

Administration of taurine or chloroamine to activated macrophages *in vitro* inhibits the generation of nitric oxide, prostaglandin E, tumor necrosis factor, and interleukin, thus modulating the inflammatory response. N-Chlorotaurine can down-regulate the generation of inflammatory mediators such as superoxide and nitric oxide.

It seems that one of the most important functions of N-chlorotaurine and taurine is to restrict cytotoxicity of the neutrophile, setting limits to the amount of damage done during inflammation.

Taurine analogues that might better suppress inflammatory responses can provide an extra dimension in treatment of inflammatory diseases. A large number of taurine analogues (Pokhrel and Lau-Cam, 2000) were tested against erythrocyte membrane damage induced by hydrogen peroxide. Many of the compounds reduced the loss of K^{+} when fresh rat erythrocytes were exposed to the action of hydrogen peroxide by as much as 47%. CA, TAU, ASBA, ACES, HMTAU, and EDSA (Table 2) showed marked attenuation (over 40% reduction) and AESA, ESA, ISA, and HYTAU only moderate attenuation (between 30 and 40% reduction).

Table 2.

Compd	Structure	Compd	Structure
TAU		AMSA	
HYTAU		ESA	
HMTAU		EDSA	
NMTAU		ISA	
MESA		ASBA	
CA		NACYS	
BALA		ACES	

2.7. Anticraving Effect

Taurine exerts its inhibitory effects on neuronal activity by potentiating the GABA and glycine receptor functions and inhibiting excitatory amino acid receptors. Taurine also modulates the Ca^{2+} channel function in the same way as ethanol does. These similarities contribute to the synergistic effects of taurine plus ethanol on the CNS. The exact mechanism is not yet solved and data have been presented that taurine antagonizes certain behavioral effects of ethanol (Oliver, 2000). Chronic administration of ethanol in experimental animals increased the brain taurine content. Recent studies have linked genetic influences on ethanol-stimulated taurine release in the CNS. Taurine plays also a role during ethanol withdrawal. During acute withdrawal from chronic alcohol intoxication, CNS neurons become hyperexcitable. Hence, the organism is prone to convulsions. Beneficial effects of taurine during ethanol withdrawal are partially due to its anticonvulsant effects as an agonist of GABA receptor. Along with taurine, some of its related compounds like cysteine and D-pantethine alleviate the circulating ethanol concentration. Administration of taurocholic acid (50 mg/kg, i.p.) 30 min prior to a narcotic dose of ethanol (5 g/kg, i.p.) reduced the time required for the onset of ethanol narcosis. Taurocholic acid (100 mg/kg, i.p.) also decreased the intake of ethanol solution in rats. Other taurine-related compounds show similar activities. One of them is acamprosate (calcium salt of N-acetylhomotaurine), a potent anticraving agent.

Successful experimental studies with acomprosate paved the way for its clinical use. Acomprosate was efficacious in reducing relapses to alcohol drinking following

detoxification. This compound is currently used to treat alcohol abuse and alcoholism (Ward *et al*., 2000). The development of acomprosate is a great success. However, further analogues with a better and long-lasting activity on various aspects of alcoholism still await development.

2.8. Bile Salt Formation

The gall bladder is the storehouse of bile, which contains various bile acids. The bile acids act as detergents to solubilize or emulsify fats transferring them to digestible components. The detergent action is due to the presence of both lipophilic and hydrophilic centers in the bile acids. Hydrophilic regions include sulfonates or carboxylates on cholesterol backbones. In almost all mammals taurine is the major amino acid, which conjugates with cholesterol derivatives to form bile salts. Taurine conjugates to form taurocholate and glycine forms glycocholate. The ratio between tauro- and glycocholate in humans is about 3:1 and this ratio is adversely affected in states of low taurine supply. Extraction of cholesterol from the body is generally through taurocholic acid. A reduced taurine supply will result in lower quantities of taurocholic acid and will severely affect the rate of extraction of cholesterol leading to its accumulation. Any agent that will increase the availability of taurine will make cholesterol more extractable. Ursodeoxycholic acid (UDCA) is widely used as cholesterol gall store-dissolving agent (Pellicciari *et al*., 1985). UDCA conjugates preferentially with glycine, because of the reduced availability of taurine in hepatocytes. This is a negative aspect since the taurine conjugate of UDCA is believed to have superior cholesterol-solubilizing properties. Administration of UDCA together with taurine has shown to form the taurine conjugate TUDCA.

2.9. Liver Protection

The liver has a major role in mammalian taurine metabolism. It is the organ where taurine synthesis and its conjugation with bile acids take place. It is critical to the maintenance of the hepatic bile flow. Taurine has shown a cytoprotective effect in the guinea pig liver. Oral administration of taurine prevented cholestasis caused by lithocholic acid sulfate and liver damages caused by carbon tetrachloride. Taurine showed chemopreventive activity against the colonic and hepatic cancer and has beneficial effects on acute hepatitis (Reddy *et al*., 1993). N-Phenylalkyltaurine (TAU-15) in a dose of 25 mg/kg provides liver protective action in rats in models of chronic toxic hepatitis and partial hepatectomy. This activity results from the antioxidative nature of TAU-15 (Khnychenko *et al*., 2001) and its ability to normalize protein synthesis in hepatocytes.

2.10 Cancer Prevention

Modifying nucleic acid structure via interaction of sulfur substances with guanosine is now a century old story. Sulfites, thiosulfates, and sulfonic acids interact with RNA and DNA and exert their protective effects against alkylating agents, radiation, and other carcinogens. Such reagents may participate in the regulation of the conformational state of nucleic acids, thus alleviating the toxic effects of chemical mutagens. Taurine, being also a sulfonic acid, can behave in a similar way. Indeed, it was shown that taurine has

radioprotective properties and antimutagenic effects, reducing various type of damage to DNA (Laidlaw *et al*., 1989; Desai et al., 1992). Cancer prevention via antioxidative mechanisms has been discussed by many researchers.

Taurine also showed to be chemoprevention against colonic and hepatic cancers. 1-(2-Chloroethyl)-3-(2-dimethylsulfonyl)ethyl-1-nitrosourea TCNU, tauromustine) has found clinical utility (Molineux *et al*., 1987). Further taurine derivatives can be designed and may find use as anticancer agents.

2.11. Stimulation of Bone Formation and Inhibition of Bone Loss

Bone is a specific tissue, which along with cartilage constitutes the skeletal system. The major functions of bone are to provide mechanical support for vital organs, bone marrow protection, and the metabolic role as a reservoir of calcium and phosphate ions, which is vital for maintenance of serum homeostasis. Like other connective tissues, bone tissue has cells and extracellular matrix. The bone matrix is composed of collagen fibers and non-collagenous proteins. However, the matrix in teeth can be calcified.

Taurine content is high in bone tissues. A strong stimulating role of taurine in bone matrix formation and collagen synthesis has been shown in osteoblast-like UMR-106 cells. Inhibition of bone resorption and osteoclast formation by taurine was also identified. Thus, taurine is a doubly beneficial agent, stimulating bone formation and inhibiting bone loss (Gupta and Kim, 2003a,b). Several taurine analogues were also evaluated for beneficial actions. For example, a combination of taurine and cystine, cysteine or methionine has protective effects in the therapy of injuries after tooth extraction in guinea pigs (Ito *et al*., 1985). Several taurine analogues showed stimulated bone formation (unpublished data). Currently, it has been shown that bisphosphonates can cure osteoporosis. Aminobisphosphonate is effective in inhibiting alveolar bone loss. Development of taurine analogues along these lines and synthesis of proper aminoethanesulfonate derivatives may provide similar inhibiting action on bone loss.

2.12. Antidiabetic and Antiaging Actions

Diabetes, though known for ages, is becoming a very common human disease. Its appearance is catalyzed by stressful life style, changing food habits, and degrading environment protection. Taurine-diabetes interaction is deeply rooted and taurine is involved in the protection of insulin-producing mechanisms. Insulin and taurine act in symbiosis, stimulating each other actions with hypoglycemic effects. Taurine alleviates further complicacy of diabetes while exerting beneficial nephropathy and retinopathy effects. Taurine supplementation to patients with type 2 diabetes mellitus provides beneficial effects (Chauncey *et al*., 2003). In the treatment of type 2 diabetes, the only effective agents belong to the family of sulfonylureas. Recently, sulfur-containing thiazolidinediones were used but with some of complicacy. Taurine-containing somatostatin compounds have been found to be effective in increasing the basal secretion of insulin in rats. Several antidiabetic formulae containing taurine are available in the market. As taurine is also a sulfonyl compound, it might be interesting to prepare and test sulfonylurea derivatives of taurine.

Ageing is a complex process affected by genetic, psychological, environmental and socioeconomic factors. All these factors influence the individual health and index of ageing. Taurine concentration declines with advancing age in spleen, kidney, eye,

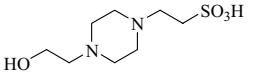
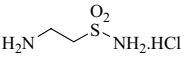
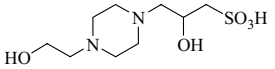
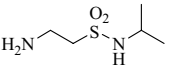
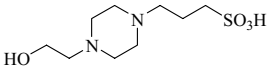
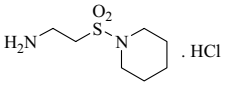
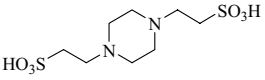
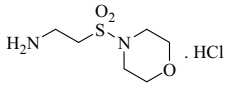
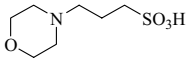
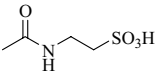
cerebellum, and blood serum. Taurine supplementation in experimental animals can correct this decline to some extent. Dietary taurine supplementation also blunted the age-related decline in serum IGF-1, increased serum creatine, and blood and urine nitrogen, thus providing beneficial effects in advanced age (Dawson, 2003).

The majority of neurodegenerative diseases are due to elevated oxidative stress and reactive oxygen species (ROS) on which taurine has scavenging properties. Advanced problems with age are also linked with the availability of glutathione. As taurine and glutathione have the same precursor, namely cysteine, the link to taurine is obvious. Several antiaging formulas containing taurine are available in the form of tablets, health drinks, lotions, etc (Seid *et al*., 2000). Development and study of taurine analogues may produce more effective antiaging agents.

2.13. Taurine Analogues in Taurine Uptake

Many taurine analogues (Rebel *et al*., 1994; Petegneif *et al*., 1995) (e.g., hypotaurine, N-methyltaurine, HEPES, ACES, TES, BES, HEPPSO, HEPPJ, EPPS, MOPS, PIPES, N-acetyltaurine, NCS 713, 2-aminobenzenesulfonic acid, 4-aminobenzenesulfonic acid, ST31-84, NCS 707, NCS 707, TAG, NCS 731, MY-117; β-alanine, and α-sulfo- and N-dimethyl-N-methylhypotaurine) (some of them shown in Table 3**)** modulate taurine uptake in cultured glial cells via two different mechanisms. The first mechanism needs the amine function of the analogues for activity, while the sulfate group can be replaced by the sulfonic group. For the second mechanism, both the amine and the sulfonate groups are required.

Table 3.

Compd	Structure	Compd	Structure
HEPES		ST-31-84	
HEPPSO		NCS 703	
HEPPS		NCS 713	
PIPES		NCS 707	
MOPS		N-Acetyltaurine	

2.14. Potential Analogues

A large number of compounds under the name of taurine analogues/derivatives are mentioned in the literature. Most of these compounds still lack biological characterization. Some are very promising, however. Biological actions are still lacking but they deserve to be mentioned. To name few, a series of 1-aryl- and 2-aryltaurines and taurinamides have been synthesized (Covello *et al*., 1970) (Fig. 8).

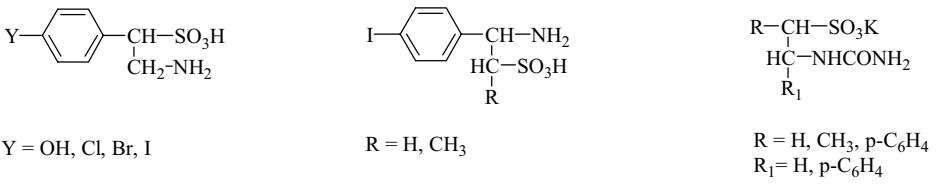


Figure 8. Substituted aromatic taurine derivatives.

β-Sultams (Fig. 9) provide taurine or substituted taurine upon hydrolysis. Such analogues have been synthesized in the past (Champsexi *et al*., 1985), but several such compounds should be designed, prepared and evaluated for taurine-like actions.

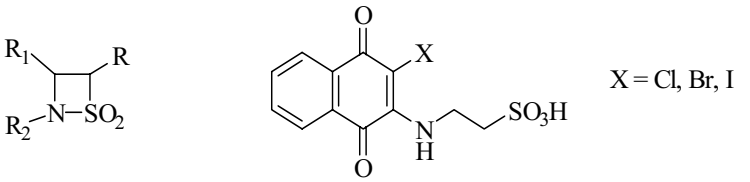


Figure 9. β-Sultams and N-quinonyltaurines.

Recently, a group of N-quinonyltaurines have been synthesized (Fig. 9) (Gorohovsky *et al*., 2003). In general, quinones can bind to a variety of bioactive molecules enhancing various biological effects. Quinones are also known as generators of ROS while taurine has an anti-ROS activity. Thus, it is expected that such N-quinonyltaurines will show interesting symbiotic effects or regulating effects, resulting in very interesting biological activities.

2.15. Inputs for Future

Biological systems are regulated by a balance between opposing influences, i.e. by the balance of inhibitory and excitatory actions. To date, a wide variety of taurine analogues/derivatives have been synthesized and studied for taurine like activity. Some were like taurine, many had even better activity and others had no activity or even showed anti-taurine effects. At the present stage it is impossible to relate the chemical

structure with the biological activity. Moreover, one analogue can act as taurine in one system and show anti-taurine activity in another system. Thus, it is yet impossible to postulate a general hypothesis for all these actions. For future design of new reactive analogous, the following guidelines will be thought.

- When designing a new analogue, it is essential that the sulfur will be in the form of a sulfonate, sulfone, or sulfonamide moiety.
- The recommended distance between the nitrogen of the amino group and the sulfur atom of the sulfonate group is two saturated carbon atoms.
- Variation, substitution, and manipulation within the methylenic groups might lead to very active analogues.
- If carbocyclic analogues are required, it is suggested to place the amino and sulfonate groups in an exo position to the ring.
- Incorporation of the amino group in a heterocyclic system will not have adverse effect on activity.
- Bulky substitution on the amino or sulfonic group might lower or annul the activity.
- Proper modification of the amino group.
- Derivatives of β-sultam and γ-sultone (cyclic homotaurine analogs) are an excellent target to develop.

These are only logical guidelines abstracted from the available literature. Of course, creative minds might open new directions, which are not on the basis of the above guidelines.

3. CONCLUSION

It is now well established that the extensive involvement of taurine is due to its unique physicochemical character derived from being a β-aminosulfonic acid. Taurine deficiency is the cause of several abnormalities, e.g, in retinal and early neural development. On the clinical front, taurine has been patented for use in acute hepatitis, congestive heart failure, epilepsy, anti-hypertension, diabetes, and more. Because of its permeability and almost lipophobic nature, transport of taurine is limited and taurine therapy requires high doses and long duration. It is thus used mainly as a supplementary agent rather than a core. Those are the reasons for the extensive search for pro-drug of taurine. Indeed, during the last 25 years a large number of analogues have been synthesized and evaluated, some with a great success. Many analogues with bioactivity as neurological or retinal protection, anti-alcohol activity, and anti cancer effects were found. A series of analogues with cyclic, acyclic and other structural variations have been developed and proved to act in retinal protection. Taurine nitrosourea is used clinically as anti-cancer agent. Another achievement of the research is the development of acamprosate, an anti-craving agent, which is widely used for treatment of alcoholics. Taurine analogues are also used against diabetic and helping in the recovery of bone loss with stimulation of bone formation

In the absence of potent taurine antagonists, we still do not know what exactly taurine does how and where it acts. This hampers development of suitable pro-drugs. Metabolic studies of analogues are missing but these are also an urgent need. As taurine has beneficial action in diabetes and ageing, efforts must be made to develop more

analogues showing better activity. As map is not the total boundary so to this, much more can be further added to make it perfect, but perfection is the end of search and creativity. However, this growing field of research needs expansion and enlargement.

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TAURINE, TAURINE ANALOGUES, AND MITOCHONDRIAL FUNCTION AND DYSFUNTION

M. Palmi,* G. Davey, K. F. Tipton, and A. Meini

1. INTRODUCTION

It has become apparent in recent years that mitochondria are integrally involved in the mechanism of both apoptosis and necrosis (Ankarcrona *et al*., 1996; Tatton *et al*., 1999; Finkel *et al*., 2001). Mitochondria possess a latent mechanism, called the permeability transition pore (PTP), that when activated will open and allow the efflux of components that lead to cell death. The PTP is known to consist of the voltage dependent anion channel (VDCA) and the adenine nucleotide transporter (ANT) present on the outer and inner mitochondria membrane respectively, and a number of other associated proteins (Duchen, 2004).

Under certain conditions such as high Ca^{2+} concentrations in the mitochondrial matrix, these proteins will undergo a conformational change, i.e. PTP opening. During this process the mitochondrial tranmembrane potential ($\Delta\Psi_m$) decreases, solutes with a molecular weight less than 1500 move into the matrix and cytochrome c (cyt c) is released into the cytoplasm where it subsequently activates the cascade of caspase enzymes. Other well-documented consequences of the PTP opening include mitochondrial swelling and decreased ATP synthesis, which all together concur in switching on the apoptotic pathway (Madl and Burgesser, 1993; Cassarino *et al*., 1999*b*; Kaufmann and Hengartner, 2001). It has been proposed that neuronal mitochondria can undergo a permeability transition under pathological conditions, which would suggest that the PTP might be involved in neuronal death in such neurodegenerative disorders as Parkinson's disease and Alzheimer's disease (Cassarino *et al*., 1999*a*; 1999*b*).

All together these data allow to hypothesize that potentiating Ca^{2+} sequestration into mitochondria matrix is detrimental for mitochondria exposed to Ca^{2+} stress conditions, since it might exacerbate the, already extensive increase in intra- matrix Ca^{2+}. However

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mitochondrial Ca^{2+} transport functions as a metabolic signal for energy production (Cormack *et al.*, 1990; Nicholls and Ferguson, 1992; Hansford, 1994) and contributes to Ca^{2+} sequestration into mitochondria (Gunter *et al.*, 1994; Kiedrowski and Costa, 1995; White and Reynolds, 1997). Therefore potentiation of Ca^{2+} sequestration might be also beneficial against Ca^{2+}-induced mitochondrial dysfunction since by enhancing the buffering capacity of the cell, protects cytosol against Ca^{2+} overload.

In line with these data, spermine and other aliphatic polyamines which enhance Ca^{2+} uptake by liver or heart mitochondria, have been shown to inhibit PTP opening (Rustenbeck *et al.*, 1998). Nevertheless, taurine (2-aminoethanesulphonic acid), a naturally occurring amino acid which also potentiates Ca^{2+} sequestration by liver mitochondria (Palmi *et al.*, 1999) and antagonizes Ca^{2+} release induced by neurotoxins (Palmi *et al.*, 1998), fails to reverse PTP opening induced experimentally by high calcium plus phosphate concentrations (Palmi *et al.*, 2000). Even tough the spermine and taurine data seem contradictory, excluding a role for the latter in regulating PTP opening, the far-from physiological Ca^{2+} conditions used in the taurine study raise the possibility that effect on PTP can not be observed at gross Ca^{2+} insult level and that more mild conditions known to increase the probability of PTP opening are necessary to see the anti-apoptotic effect of taurine. To investigate on this possibility, we studied the role of taurine and some taurine analogues on function of mitochondria exposed to Ca^{2+} concentrations ($[Ca^{2+}]$) ranging between 5 and 50 μM. Changes in mitochondrial volume, $\Delta\Psi_m$ and cyt c release were used as biochemical markers of mitochondrial function.

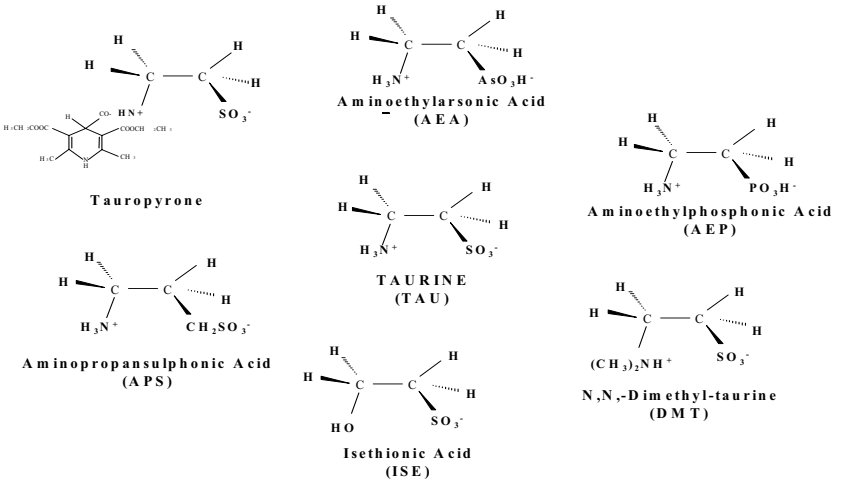

Figure 1. Structures of taurine and taurine analogues.

2. MATERIALS AND METHODS

2-Aminoethylarsonic acid (AEA) and *N,N*-dimethyltaurine (DMT) were prepared as previously described (Palmi *et al.*, 1999). Tauropyrone was a generous gift from Prof. Tirzitis, Institute of Organic Synthesis, Riga, Latvia. 2-Hydroxyethanesulphonic

(isethionic) acid (ISE), 3-aminopropanesulphonic acid (APS), 2-aminoethylphosphonic acid (AEP), and all other chemicals were from Sigma (Milan, Italy).

The structures of taurine and the taurine analogues used in this work are presented in Fig. 1.

2.1. Mitochondrial Preparation

Male Wistar rats (200-250 g) were housed under standard conditions with food pellets and water *ad libitum*. For all animal experiments described in this study, the approval was obtained from the University of Siena Ethic Committee (D.L. 116/92). After a light anesthesia with a mixture of Ketavet® and Rompun® (60 and 16 mg/kg b.w., respectively) the rats were killed by decapitation and rapidly exsanguinated. The livers were rapidly removed and homogenized in 250 mM sucrose, 5 mM Tris, 1 mM EGTA [(ethyleneglycol-*bis*(2-aminoethylether)-*N,N,N'N'*-tetraacetic acid)] (STE) at pH 7.4, and mitochondria were separated by differential centrifugation (Chappel and Hansford, 1972). After centrifugation, the mitochondrial pellet was resuspended in 1-2 ml of STE, pH 7.2. The mitochondrial protein content was determined by the biuret method (Gornall *et al*., 1949) using bovine serum albumin (BSA) as standard.

2.2 Measurement of $\Delta\Psi_m$ in Isolated Mitochondria

In order to measure the relative change in $\Delta\Psi_m$, the uptake of JC-1, a membrane potential sensitive fluorescent probe, was assessed. JC-1 is a cationic dye that exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (JC-1 monomer fluorescence, λ_{em}=525 nm) to red (J-aggregate fluorescence, λ_{em}=590nm) (Smiley *et al*., 1991).

Mitochondria (0.25 mg/ml) were suspended in a cuvette containing 120 mM KCl, 10 mM HEPES [*N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid)], 10 mM glutamate, 5 mM malate, 1 mM EGTA, 5 mM KH_2PO_4, pH 7.2 and incubated for 5 min at 37°C in the dark with JC-1 (1 μM). The cuvette was placed in a sample compartment of a spectrofluorophotometer (RF-5000, Shimadzu) equipped with a magnetic stirrer and a temperature controlled cuvette holder. The emission spectrum of JC-1 was monitored at λ_{ex} = 490 nm, between λ_{em} = 515 and λ_{em} = 615 nm and repeated at intervals of 1 min. When spectrum was stabilized, $CaCl_2$ was added and calibrated with 1 mM EGTA present in the buffer (Maxchelator; Dr. C. Patton, Standford University, Standford, CA) to give a final free [Ca^{2+}] of 5, 10, 25, or 50 μM.

Depolarization of $\Delta\Psi_m$ was estimated 5 min after addition of Ca^{2+} and expressed as red to green fluorescence ratio. When the effect of taurine on Ca^{2+}-induced changes in $\Delta\Psi_m$ was investigated, the samples were supplemented with 1 mM taurine 10 min before Ca^{2+}addition.

2.3. Measurement of Mitochondrial Swelling

Mitochondrial swelling was measured, following the method of Broekemeier *et al*. (1989), by monitoring the decrease in the light-scattering of a mitochondrial suspension. Following preparation in STE, the mitochondria were suspended in incubation medium containing 120 mM KCl, 20 mM HEPES, 10 mM glutamate, 5 mM malate, 1 mM

EGTA, 5 mM KH_2PO_4, pH 7.2, and placed in a 3 ml stirred cuvette at 25°C. The final suspension containing 1 mg/ml of mitochondria was monitored spectrophotometrically at 540 nm in a Shimadzu UV-160 spectrophotometer. After the attenuance stabilized, taurine (1 mM) or taurine analogues (1 mM) were added, followed 10 min later by $CaCl_2$ calibrated with the 1mM EGTA to give a final free [Ca^{2+}] of 5, 10, 25, or 50μM.

2.4. Measurement of Cytochrome c Release by Western Blot Analysis

Mitochondria (2.5 mg/ml) were suspended in medium consisting of 120 mM KCl, 10 mM HEPES, 10 mM glutamate, 5 mM malate, 1 mM EGTA, 5 mM KH_2PO_4, pH 7.2 and loaded with $CaCl_2$ (5, 10, 25, or 50 μM) with or without taurine (1 mM), and then incubated for 10 min at ambient temperature. After centrifugation at 15000g for 2 min at room temperature, 20 μl of supernatant was removed from each sample, supplemented with 4x loading buffer, [(1 M Tris-HCl, pH=6.8, 10% dithiothreitol (DTT), 10% sodium dodecylsulfate (SDS), glycerol, blue bromophenol)] and boiled for 2 min at 95-100°C.

The samples and horse heart cytochrome c (cyt c) as a positive control were loaded onto 10% SDS-PAGE and separated by the molecular size. Prestained rainbow markers (Amersham Biosciences, Milan, Italy) were used as molecular mass standards. The gels were then electroblotted onto nitrocellulose membranes.

Immunodetection of the protein of interest was performed by blocking first the membrane in 5% nonfat dry milk (VWL International) in 20 mM Tris-base, 140 mM NaCl, 0.05 % Tween 20 (TBS-Tween 20), pH 7.4, for 2 h at room temperature. After two washes in TBST, the blots were probed with rabbit anti cyt c antibodies to detect cyt c (1:750; Oncogene, Milan, Italy). Antibody binding to protein was detected, using anti-rabbit secondary antibodies conjugated with horseradish peroxidase (1:2500, Promega, Milan, Italy) and visualised autoradiographically on film, using enzyme-linked chemiluminescence (ECL, Amersham Biosciences, Milan, Italy).

2.5. Statistics

The mean and SEM values from 3 to 5 separate experiments were determined and the differences between these and controls calculated. The statistical significance of the differences was determined by Student's *t*-test for paired data followed by Welch's *t*-test.

3. RESULTS

3.1. Effect of Taurine and Some Taurine Analogues on Mitochondrial Swelling

Opening of the permeability transition pore (PTP) to induce mitochondrial swelling can be caused by such physiologic compounds as Ca^{2+} and inorganic phosphate (P*i*) (Kowalkowski *et al*., 1996). Therefore, experiments were performed to ascertain whether under our Ca^{2+} and P*i* conditions mitochondrial swelling occurred and in such a case the role played by taurine or some taurine analogues. In the presence of 5 mM KH_2PO_4, varying concentrations of Ca^{2+} were added to the mitochondrial samples, and after a period of stabilization of 1-2 min, changes in absorbance were monitored.

As Fig. 2 (panel A) shows, the addition of 5 μM Ca^{2+} induced a prompt swelling of mitochondria, revealed by decreased absorbance (50.4% ± 4.8 with respect to control)

after a period of 3-4 min. This effect was antagonized significantly ($p<0.01$) by pretreatment with 1 mM taurine. Controls treated with taurine in absence of Ca^{2+}, presented a slight and time-dependent increase in absorbance which nevertheless did not differ significantly from controls. When the same experiment was repeated using 10, 25, or 50 μM Ca^{2+}, taurine failed to counteract swelling (Fig. 2, panels B, C and D respectively), suggesting the Ca^{2+} dependence of taurine effect. The finding that cyclosporin A (CsA) prevented mitochondrial swelling induced by the highest [Ca^{2+}] (Fig. 2, panel D) suggested that under our experimental conditions swelling was mediated by PTP opening.

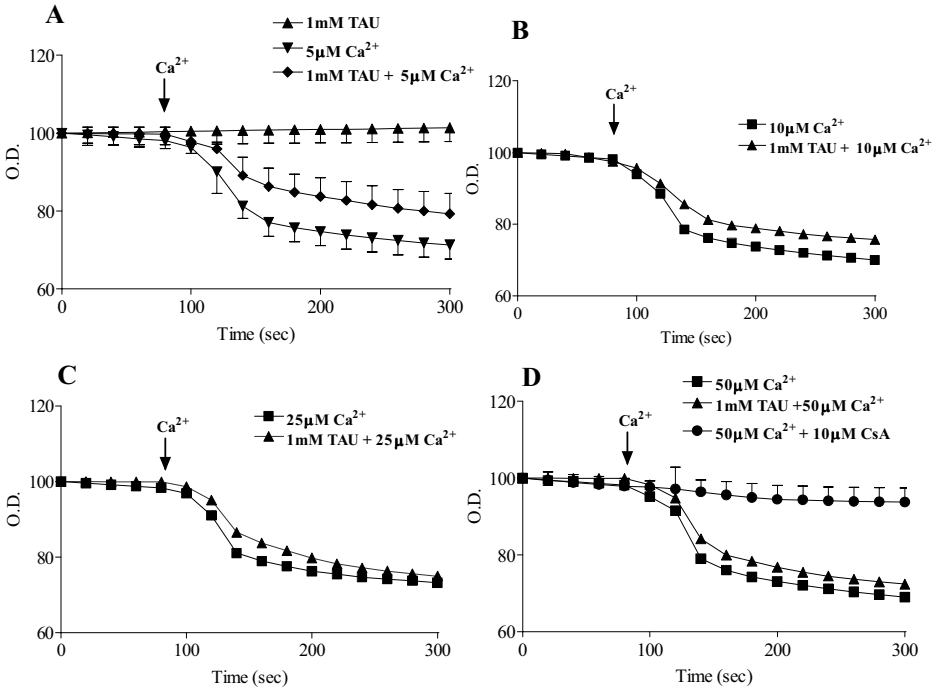

Figure 2. Calcium dependence of taurine effect on mitochondrial swelling. Freshly isolated mitochondria from rat liver (1 mg/ml) were incubated in a P*i* and glutamate/malate-containing medium in the absence or presence of taurine. After the attenuance stabilized, 1 mM taurine was added, followed 10 min later by $CaCl_2$ calibrated with EGTA to give a final free Ca^{2+} concentration of 5 (panel A), 10 (panel B), 25 (panel C) or 50 μM (panel D). Changes in volume of mitochondrial suspensions were monitored with time by measuring the decrease in absorbance at 540 nm. All traces represent the means ± SEM from 3-5 separate experiments. Control (100%) represents the average absorbance from values obtained in the 0-80 sec interval preceding Ca^{2+} addition (see arrow). Group data of absorbance from each treatment were compared statistically by Student's t test across all treatment conditions. A $p < 0.05$ was considered statistically significant. Not significant differences between groups are not specified. Control *vs.* Ca^{2+} (5 μM), $p< 0.001$; Ca^{2+} (5 μM) *vs*. Ca^{2+} (5 μM) + taurine (1 mM), $p < 0.01$.

To assess whether taurine effect on Ca^{2+}-induced swelling was selective, the same experiment was repeated with different taurine analogues. As shown in Fig. 3, in the presence of 5 μM Ca^{2+}, 1 mM AEA, ISE, APS, AEP, or tauropyrone had no significant effects, whereas DMT prevented Ca^{2+} -induced swelling.

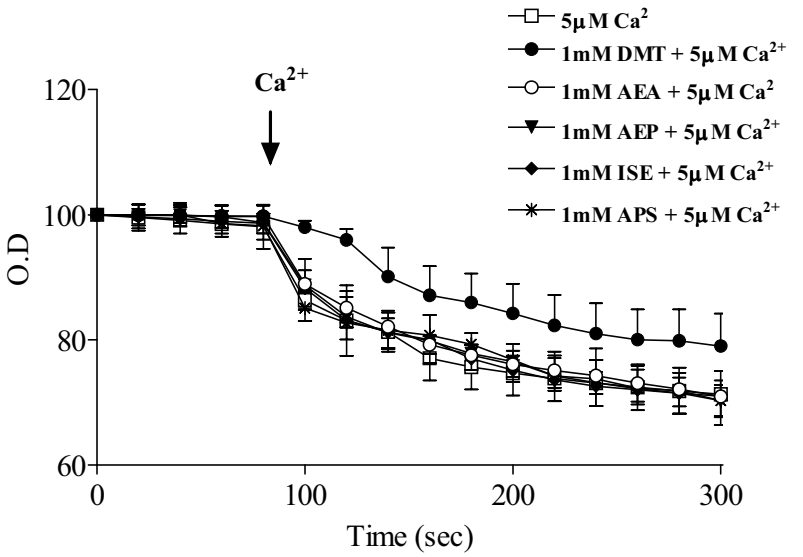

Figure 3. Effect of taurine analogues on mitochondrial swelling induced by Ca^{2+} excess Freshly isolated mitochondria from rat liver (1 mg/ml) were incubated in a P*i* and glutamate/maleate containing medium in the absence or in the presence of various taurine analogues. After the attenuance stabilized, *N,N*-dimethyltaurine (DMT), 2-aminoethylarsonic acid (AEA), isethionic acid (ISE), 3-aminopropanesulphonic acid (APS), 2-aminoethylphosphonic acid (AEP), or tauropyrone were added at a concentration of 1 mM followed 10 min later by 5 μM $CaCl_2$. For details see the legend to Fig. 1. Control *vs.* Ca^{2+} (5 μM), $p< 0.001$; Ca^{2+} (5 μM) *vs.* Ca^{2+} (5 μM) + DMT, $p < 0.01$.

3.2. Effect of Taurine on $\Delta\Psi_m$

Fig. 4 shows that in the presence of P*i*, the addition of Ca^{2+} to the experimental medium induced depolarization of $\Delta\Psi_m$ as revealed by decrease in 590/525 nm fluorescence ratio. Even though 5 μM Ca^{2+} affected $\Delta\Psi_m$ by reducing it to 65.4% ± 2.8 of control, the progressive increases in [Ca^{2+}] did not induce further statistically significant decline of $\Delta\Psi_m$. Pretreatment with 1 mM taurine significantly antagonized the drop of $\Delta\Psi_m$ induced by 5 or 10 μM Ca^{2+} but not that induced by 25 or 50 μM Ca^{2+}. Controls treated with taurine alone presented a slight increase in fluorescence ratio, which nevertheless did not differ significantly from that of controls. In contrast, CsA completely antagonized the loss of potential induced by the highest [Ca^{2+}]. In line with the swelling data, these findings indicate that taurine has a protective role against Ca^{2+}-induced mitochondrial depolarization and that this effect is Ca^{2+}-dependent.

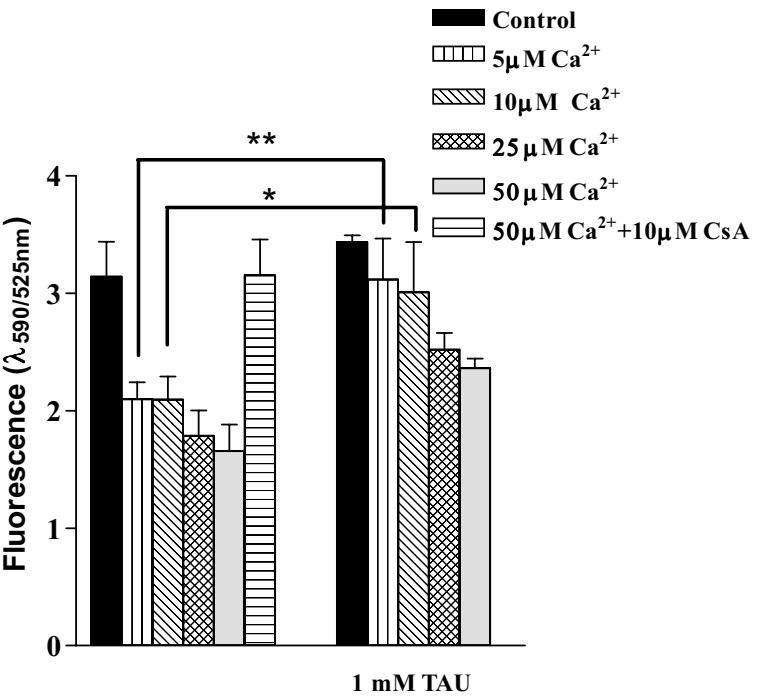

Figure 4. Calcium-dependence of taurine effect on mitochondrial $\Delta\Psi_m$ In order to measure changes in $\Delta\Psi_m$, the uptake of JC-1 into mitochondria was assessed. Freshly isolated mitochondria from rat liver (0.25 mg/ml) were suspended in a P*i* and glutamate/maleate-containing medium and incubated for 5 min in the dark with JC-1 in the presence or absence of taurine. $CaCl_2$ was added and calibrated with 1 mM EGTA to give a final free Ca^{2+} concentration of 5, 10, 25, and 50 μM. Depolarization of $\Delta\Psi_m$ was estimated as red (λ_{em} = 590 nm) to green (λ_{em} = 525 nm) fluorescence ratio. One mM taurine was supplemented 10 min before Ca^{2+} addition.
* $p < 0.05$ and ** $p < 0.01$.

3.3. Effect of Taurine on Release of Cytochrome c From Mitochondria

It is known that swelling leads to rapture of the inner and outer mitochondrial membranes that release factors such as cyt c and AIF, which are thought to induce apoptotic degradation of the cell (Tatton and Olanow, 1999). Therefore, experiments were performed to ascertain whether under our Ca^{2+} and P*i* conditions mitochondria released cyt c and in such a case the role played by taurine.

Fig. 5 shows that in absence of Ca^{2+}, cyt c was not released from mitochondria. Under the same conditions, taurine had no effect on release of cyt c. In contrast, addition of 5, 10, 25, and 50 μM Ca^{2+} induced release of cyt c from mitochondria. Pretreating samples with 1 mM taurine prevented the release of this protein but only when mitochondria were exposed to [Ca^{2+}] not exceeding the 10 μM level.

Also in line with previous findings, CsA completely prevented cyt c release induced by the highest [Ca^{2+}].

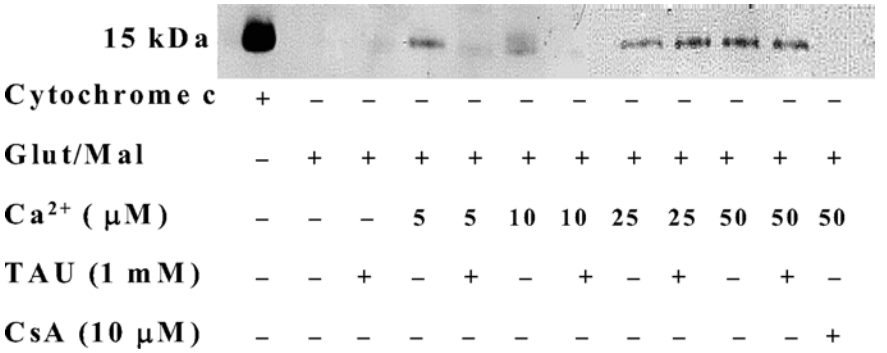

Figure 5. Calcium dependence of taurine effect on cyt c release. Cyt c in the supernatant was analysed by SDS PAGE followed by Western blot analysis. Freshly isolated mitochondria from rat liver (2.5 mg/ml) were suspended in a P*i* and glutamate/malate-containing medium loaded with $CaCl_2$ (5, 10, 25, or 50 μM) in the absence or presence of 1 mM taurine and then incubated for 10 min at ambient temperature. After centrifugation the supernatants were analyzed by Western blot analysis using anti-cyt c antibodies.

4. DISCUSSION

Previous findings by our group showed that taurine stimulates Ca^{2+} uptake by rat liver mitochondria by a selective mechanism envisaging direct stimulation of the Ca^{2+} uptake system (uniporter) rather than inhibition of Ca^{2+} efflux (Palmi *et al*., 1999). Furthermore the increased Ca^{2+} uptake promoted by taurine counteracted Ca^{2+} release from mitochondria induced by a combined treatment with the neurotoxins 1-methyl-4-phenylpyridinium (MPTP) and 6-hydroxy dopamine (Palmi *et al*., 1998).

Whereas the effect of taurine on Ca^{2+} uptake is potentially protective towards moderate elevation of $[Ca^{2+}]_i$, under far-from physiological Ca^{2+} conditions, the same taurine effect might exacerbate the increase, already extensive, in intra-matrix Ca^{2+}. Thus under these extreme conditions, taurine might not be expected to be protective but to enhance the probability of PTP opening. Our present data on isolated, functionally intact liver mitochondria exposed to graded Ca^{2+} levels showed that this was indeed the case. Therefore, the parameters indicative of the presence of PTP opening such as swelling, drop in $\Delta\Psi_m$, and cyt c release, all converged to indicate that taurine protected mitochondrial function against a limited elevation of $[Ca^{2+}]$, which we found to correspond to a threshold level of 5 μM. In line with these data, we recently showed that taurine failed to reverse changes in mitochondrial swelling, cyt c release, and $\Delta\Psi_m$ induced by 50 μM Ca^{2+} (Palmi *et al*., 2000).

Among the taurine analogues tested, DMT shared the taurine property, protecting mitochondria against swelling induced by 5 μM Ca^{2+}, whereas AEP, ISE, APS, and tauropyrone had no significant effects. Finally, AEA prevented the effect of taurine on Ca^{2+}-induced swelling (data not shown). These data and those previously shown by our group on the effect of taurine analogues on mitochondrial Ca^{2+} uptake provide evidence of a strict relationship between potentiation of Ca^{2+} uptake and protection of mitochondrial function. Indeed, DMT increases Ca^{2+} sequestration into mitochondria while AEP, ISE, and APS are ineffective. Furthermore, even though AEA is a weak

inhibitor of Ca^{2+} uptake, it inhibits the uptake stimulated by taurine (Palmi *et al*., 1999). All together these findings strongly support the notion that the mechanism underlying the protective effect of taurine against PTP opening is selective and related to the role played by this amino acid on mitochondrial Ca^{2+} transport.

The finding that the protective function of taurine is dependent on the degree of Ca^{2+} insult may account for the contradictory data in literature, where the potentiation of mitochondrial Ca^{2+} uptake by bcl-2 over-expressing neural cells has been alternatively reported to prevent or potentiate cell death induced by apoptotic insults (Murphy *et al*., 1996; Kruman *et al*., 1999). Similarly, spermine and other aliphatic polyamines that enhance Ca^{2+} uptake by liver or heart mitochondria inhibit, but do not abolish, the PTP-mediated Ca^{2+} release induced by an acute increase in Ca^{2+} concentration in a cytosol-adapted incubation medium (Ca^{2+} pulse) (Rustenbeck *et al*., 1998).

Taurine was found to interact with the neutral phospholipids in biological membranes thereby altering membrane architecture, fluidity, and properties such as ion channel functions, membrane-bound enzyme activities, and several Ca^{2+}-dependent cellular functions, including Ca^{2+} transport (Huxtable, 1992; Nakashima *et al*., 1996). These properties may have a profound influence on the stability of the inner membrane of mitochondria and account for the effect of taurine on the PTP opening observed by us.

Potentiation of the cellular Ca^{2+}-buffering capacity promoted by taurine might be beneficial to the maintenance of normal cellular functions, particularly in those pathological disturbances characterized either by Ca^{2+} accumulation in cytoplasm or loss of cellular energy, such phenomena that occur during oxidative stress, excitotoxicity, and reperfusion following hypoxia/ischemia. Indications that damage due to these conditions might be indeed ameliorated by taurine supplementation comes from the observations that taurine counteracts the Ca^{2+} influx and overload in brain tissue caused by hypoxia (Schurr and Rigor, 1987) and that the tissue taurine level increases in different neuron-damaging conditions, including hypoxia, hypoglycemia, ischemia, oxidative stress, and the presence of free radicals and metabolic poisons (Saransaari and Oja, 2000). Accordingly, taurine prevents neurotoxicity in different cultured neurons exposed to glutamate or MPP^{+} neurotoxicity by a mechanism involving regulation of intracellular and/or mitochonrial Ca^{2+} homeostasis (O'Byrne and Tipton, 2000; Chen *et al*., 2001; El Idrissi and Trenkner, 2003; Louzada *et al*., 2004).

In conclusion, we demonstrated that taurine has a protective role on mitochondrial function under mild Ca^{2+} stress conditions. The mechanism underlying this effect relies on taurine-induced decrease of the probability of PTP opening. We also showed that this effect is selective and related to the reported property of taurine of potentiating Ca^{2+} sequestration into mitochondria matrix. Our study also indicates that under extreme Ca^{2+} levels the same taurine property to potentiate mitochondrial Ca^{2+} uptake might result in excessive matrix Ca^{2+} accumulation and detrimental effects on mitochondrial function.

Since dysfunction of mitochondria by excess of Ca^{2+} might characterize the early stage of the apoptotic process, it is likely that taurine is central in coordinating the events that control apoptotic cell death.

5. ACKNOWLEDGMENTS

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ANTI-INFLAMMATORY EFFECTS OF TAURINE DERIVATIVES (TAURINE CHLORAMINE, TAURINE BROMAMINE, AND TAUROLIDINE) ARE MEDIATED BY DIFFERENT MECHANISMS

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1. INTRODUCTION

Taurolidine (TRD) is a synthetic derivative of taurine, which is chemically designed as bis-(1,1-dioxoperhydro-1,2,4-thiadiazinyl-4)methane, originally designed as a chemoterapeutic agent. It is commonly used as adjunctive therapy for various infections (Browne *et al.,* 1976; Traub *et al.,* 1993). Recently, it has been shown that TRD exerts antineoplastic activities and inhibits the growth of a variety of tumor cell lines *in vitro* and *in vivo* (McCourt *et al.,* 2000; Darnowski *et al.,* 2004; Nici *et al.,* 2004). TRD is degraded *in vivo* into three major breakdown products (Scheme 1). Methylol-containing TRD breakdown products, taurultam and taurinamide exert anti-bacterial, anti-endotoxin and anti-adherence activities (Gorman *et al.,* 1987; Willatts *et al.,* 1995). Taurine, the third breakdown product of TRD, does not share those activities. On the other hand, it has been reported that taurine is responsible for TRD immunoregulatory properties, such as inhibition of production of pro-inflammatory cytokines (Bedrosian *et al.,* 1991; Watson *et al.,* 1995). However, the results are controversial and the contribution of taurine in TRD anti-inflammatory activity is not clear.

On the contrary, it is well documented that taurine haloamines, taurine chloramine (TauCl) and taurine bromamine (TauBr) exert immunoregulatory properties (Marcinkiewicz *et al.,* 1995; Schuller-Levis and Park, 2003; Marcinkiewicz *et al.,* 2005). As taurine derivatives, TRD and taurine haloamines, but not taurine itself, can down-regulate inflammation (Marcinkiewicz 1997; Watson *et al.,* 1995), it is reasonable to establish whether myeloperoxidase **(**MPO) halide system may contribute to TRD-

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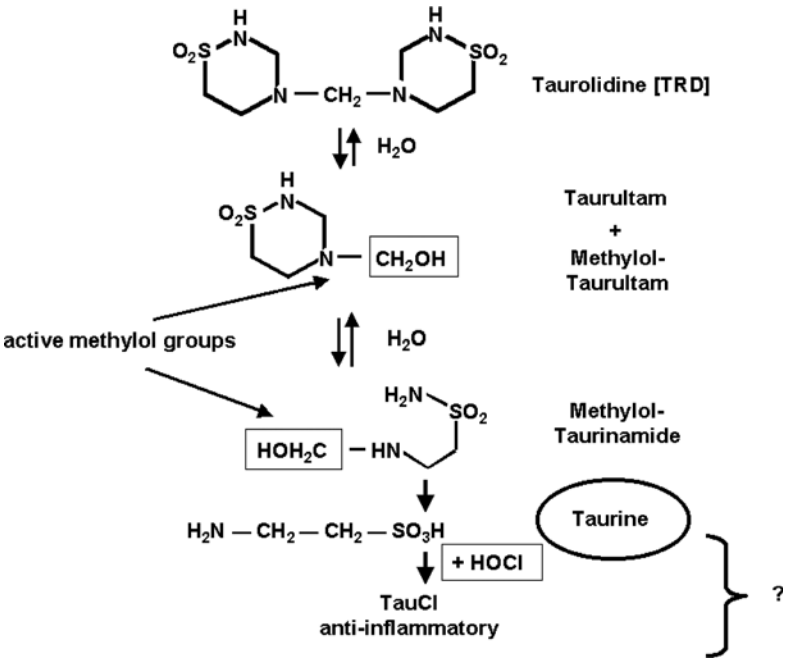
dependent anti-inflammatory activities. Therefore, the formation and biological properties of chlorinated TRD remain to be elucidated.

TauCl and TauBr, the physiological products of reaction between taurine and HOCl or HOBr, are major haloamines generated at a site of inflammation (Klebanoff and Hamon, 1979; Thomas *et al.,* 1995). Both taurine haloamines exert bactericidal and anti-inflammatory properties (Nagl *et al.,* 2000; Marcinkieicz *et al.,* 2005). They decrease the production of pro-inflammatory mediators by various types of activated cells: macrophages, neutrophils, dendritic cells, fibroblast-like synoviocytes and glial cells. TauCl was described to inhibit production of NO, PGE_2, TNF-α, IL-6, IL-8 and ROS (Marcinkiewicz *et al.,* 1995, 1999; Park *et al.,* 1995; Kontny *et al.,* 2000). Studies on the mechanism of its action have revealed that TauCl inhibits the activation of NFκB, a potent signal transducer for inflammatory cytokines, by oxidation of IκBα at methionine[45] (Barua *et al.,* 2001). More recently it has been shown that TauCl and TauBr induce expression of heme oxygenase-1 (HO-1) (Olszanecki and Marcinkiewicz, 2004), a stress-inducible protein with strong anti-inflammatory properties (Vincente *et al.,* 2003).

In our study we have addressed the issue whether TRD down-regulates acute inflammation and whether the MPO-halide system, via the formation of TRD-chlorinated species, may contribute to the anti-inflammatory properties of TRD. To clarify this problem we have tested *in vivo* the effect of local administration of TRD on the development of zymosan-induced peritonitis and *in vitro* the effect of TRD and its



Scheme 1. Structure of taurolidine (TRD) and its major breakdown products taurultam, taurinamid and taurine. Upon breakdown, TRD generates methylol-containing fragments (-CH_2OH) that have been suggested as being responsible for its anti-bacterial, anti-endotoxin, anti-adherence and pro-apoptotic activities.

chlorinated product (TRD-Cl) on cytokine production by macrophages as well as on the expression of HO-1 protein in macrophage cytosol. The effects were compared with those of TauCl. Therefore, the aim of this study was to evaluate the anti-inflammatory activities of TRD and its chlorinated product (TRD-Cl) in comparison to TauCl.

2. MATERIALS AND METHODS

2.1. Reagents

Taurolidine: For *in vivo* study Taurolin® for injections (Boehringer Ingelheim, Germany*)* 2% w/v aqueous isotonic solution of taurolidine (TRD) in 5% polyvinylpyrrolidine (PVP) was used as a source of TRD. PVP (Sigma, St Louis, MO, USA), the pharmaceutical stabilizer of TRD, was used as a control (placebo). For *in vitro* studyTRD, which has a low molecular weight (284), was isolated from Taurolin and separated from PVP (40 000 MW) by centrifugation (2000 x g, 20 min) on Vivaspin membrane 5000 MW (Vivascience, Germany). Chlorinated TRD (TRD-Cl) was prepared by a reaction of TRD with equimolar concentration of HOCl. The reaction was monitored by UV absorption spectra (λ_{max} for chloramines is 252 nm).

2.1.1. Preparation of Taurine Chloramine (TauCl)

TauCl was prepared by a dropwise addition of 5 ml of 20 mM NaOCl (Aldrich, Steinham, Germany) solution in 0.05 M phosphate buffer (pH 7.4) into 5 ml of 24 mM taurine (Tau) (Sigma, St. Louis, MO, USA), with vigorous stirring. Each preparation of TauCl was monitored by UV absorption spectra (λ = 200 to 400 nm) to assure the authenticity of monochloramine (TauCl) (λ_{max} is 252 nm) and the absence of dichloramine ($TauCl_2$) (λ_{max} is 300 nm) and unreacted $HOCl/OCl^-$ (λ_{max} is 292 nm). The concentration of synthesized TauCl was determined using the molar extinction coefficient 429 $M^{-1}cm^{-1}$ at A_{252} (Thomas *et al.,* 1986). The stock solution of TauCl was kept at 4°C for maximum 5 days before use.

2.2. Mice

Inbred Balb/c male mice from the breeding unit, Department of Immunology, Jagiellonian University Medical College, Cracow, Poland, were used between 6 and 8 weeks of age. The authors were granted permission by the Local Ethics Committee to use mice in this study.

2.3. Induction and Evaluation of Acute Peritonitis

The *in vivo* effect of TRD on the development of zymosan-induced peritonitis in Balb/c mice was investigated. Mice received i.p 1.0 mg of zymosan with either Taurolin (TRD 10 mg/kg + PVP) or with placebo (PVP, a dose equivalent to that present in the Taurolin solution). The severity of acute inflammation was determined by measurement of vascular permeability, neutrophil influx into he peritoneal cavity and proinflammatory cytokine production *in vitro* by peritoneal exudate cells.

The influence of TRD on vascular permeability was investigated by determination of Evans blue leakage from the circulation into the peritoneal cavity. Briefly, the mice received i.v 100 μl of 0.5 % solution of Evans blue 30 minutes after induction of peritonitis. After additional 30 min, the mice were sacrificed and the peritoneal cavity was washed with 1.0 ml of phosphate buffer solution (PBS). The concentration of Evans blue at peritoneal exudates was determined by measuring the optical density of cell-free exudates (λ is 630 nm). The content of neutrophils at a site of inflammation was estimated by determination of MPO activity in peritoneal exudate cells. Moreover, the effect of local treatment of TRD on cytokine production by peritoneal exudate cells (PEC) has been tested. PEC were restimulated *in vitro* with IFN-γ for 24 h. The supernatants were collected and tested for TNF-α and IL-6.

2.4. Cells

Peritoneal mouse macrophages (Mϕ) were induced by intraperitoneal injection of 1.0 ml of paraffin oil (Sigma, St.Louis, MO, USA). Cells were collected 48 h later by washing the peritoneal cavity with 5 ml of PBS, containing 5 U/ml heparin (Polfa, Warsaw, Poland). The cells were centrifuged and red blood cells were lysed by osmotic shock using distilled water; and osmolarity was then restored by addition of 2 x concentrated PBS. The presence of macrophages (85-90%) was judged by cytochemical demonstration of non-specific esterase-positive mononuclear cells, using *α*-naphtyl acetate (Sigma, St Louis, MO, US).

2.5. Cell Cultures and Treatment

Mϕ were cultured in 24-well flat-bottom cell culture plates at 5 x 10^5/well in RPMI 1640 medium (JR Scientific Inc., Woodland, CA, USA) supplemented with 5% FCS at 37°C in an atmosphere of 5% CO_2. Cells were activated with either 50 U/ml of IFN-γ (Sigma, Steiham, Germany) or 100 ng/ml of LPS (*E. coli* 0111 B:4, Sigma Steiham, Germany) and cultured in the presence of test agents. After the 24-h culture, the supernatants were collected and frozen at -80°C until used.

2.6. Measurement of Cell Viability

The viability of the cells was routinely monitored by cellular exclusion of trypan blue. In some experiments, cell respiration, an indicator of cell viability, was assessed by mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide (MTT) to formazan. Cells in 96-well plates were incubated at 37°C with MTT (0.2 mg ml^{-1} for 60 minutes). Then, culture medium was removed by aspiration and the cells were solubilized in dimethylsulfoxide (DMSO, 200 μl). The extent of reduction of MTT to formazan within cells was quantitated by measurement of absorbance at 550 nm.

2.7. Cytokine Determination

The cytokine concentrations in culture supernatants were measured using capture ELISA. Briefly, for IL-6 measurment 96-well plates (Corning, NY, USA) were coated overnight with rat mAb against a mouse cytokine (capture antibody). After blocking the

plates with 4% albumin (2 h), standards and tested supernatants were added and incubated overnight. Finally, the plates were coated with biotinylated antibodies against the same cytokine-detecting antibody for 1 h. ELISA was developed with horseradish peroxidase streptavidin (Vector, Burlingame, CA, USA), followed by *o*-phenylene-diamine and H_2O_2 (both Sigma, Steiham, Germany) as substrates for 30 min. For TNF-α, a peroxidase-conjugated goat anti-rabbit IgG (Sigma, Steiham, Germany) was used to develop the reaction. The reaction was stopped with 3 M H_2SO_4 and the optical density of each well was measured at 492 nm in a plate reader.

IL-6: Rat anti-IL-6 and biotinylated rat anti-IL-6 (both Pharmingen, San Diego, CA, USA) mAbs were used as detecting antibodies. Recombinant mouse IL-6 (PeproTech Rocky Hill, New York, USA) was used as a standard. The detection limit was about 15 pg IL-6/ml.

TNF-α: Rat anti-murine TNF-α and biotinylated rat anti-mouse TNF-α (Pharmingen, San Diego, CA, USA) mAbs were used as detecting antibodies. Recombinant mouse TNF-α (Pharmingen, San Diego, CA, USA) was used as a standard. The limit of detection was 30 pg TNF-α/ml.

2.8. Measurement of Myeloperoxidase Activity

Freshly collected zymosan-induced cells from exudates of the mice treated with either TRD or placebo (PVP, a dose equivalent to that present in the Taurolin solution) were resuspended (~10^6 cells/ml) in 0.5% hexadecyltrimethylammonium (HTAB) (Sigma, Steiham, Germany) in 50 mM potassium phosphate buffer, pH 6.0. The cells were freeze-thawed in three cycles, dispersed by vortexing and the supernatants were collected by centrifugation at 4000 x g at 10 min at 4°C. Twenty µl sample supernatants were then mixed with 180 µl of 50 mM potassium phosphate buffer (pH 6.0) containing 0.167 mg/ml o-dianisidine dihydrochloride and 0.0005% hydrogen peroxide (both Sigma, Steiham, Germany) and placed in the 96-well flat bottom plate. After 20-min incubation at room temperature the absorbance at 460 nm was measured in a plate reader. The MPO activity was calculated from a standard curve prepared the same way as test samples by using the MPO standard (Sigma, Steiham, Germany) and expressed in units per milligrams of protein. One unit of MPO activity was defined as an increase in absorbance of 1.0 per min at room temperature and the concentration of protein in samples was measured at 280 nm calculated from bovine serum albumin (BSA) standard curve. Each sample was measured in duplicate.

2.9. Western Blot Analysis: Evaluation of HO-1 Expression.

Non-stimulated Mϕ were cultured with taurine derivatives and the expression of heme-oxygenase-1 (HO-1) protein in cytosol was determined by a Western blot technique. Twenty four hours after incubation with the agents, the cells were lysed in lysis buffer [1% Triton X-100, 0.1% sodium dodecylsufate (SDS) in PBS containing 1 mM phenylmethylsulphonylofluoride (PMSF), 100 µM leupeptin and 50 µM pepstatin A]. The protein concentrations of lysates were determined using the Bradford method. The samples, containing equal amounts of total protein, were mixed with gel-loading buffer (50 mM Tris, 3% SDS, 10% glycerol, 7% 2-mercaptoethanol and 0.1% bromophenol blue) in a ratio 4:1 (v/v) and boiled (4 min). Then samples (20 µg of total protein per lane) were separated on 10% SDS-polyacrylamide gels (Mini Protean II, Bio-

Rad, USA) using Laemmli buffer system and proteins were semi-dry transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, USA). Non-specific binding sites were blocked overnight at 4°C with 5% non-fat dried milk and the membranes were then incubated for 2 h at room temperature (RT) with mouse monoclonal antibody to HO-1 (1:2000) (Stressgen, Canada). Bands were detected with horseradish peroxidase-conjugated secondary antibody (1 h in RT, 1:5000, Amersham Pharmacia Biotech, USA) and developed with ECL reagents (Amersham Pharmacia Biotech, USA). Additionally, membranes were re-probed with monoclonal anti β-actin antibody (Sigma, USA). Rainbow markers (Amersham Pharmacia Biotech, USA) were used for the molecular weight determinations. The protein bands were scanned and analyzed with freeware Scion image (Scion Corporation, USA). The data are normalized to constitutively expressed β-actin protein.

2.10. Statistical Analysis

If not otherwise stated, the statistical significance of differences between groups was analysed using a factorial ANOVA (Microsoft Excel) followed by Student's t test, if appropriate. The results are expressed as mean ± SE. A p-value less than 0.05 was considered statistically significant. All statistical analyses were performed using Statistica PL™ v. 6.0 (StatSoft, Poland).

3. RESULTS

3.1. Effect of *in Vivo* TRD (Taurolin) Administration on Zymosan-Induced Peritonitis

To evaluate the effect of TRD on the development of acute inflammatory response, vascular permeability, MPO activity of peritoneal exudates cells, which linearly correlates with the number of neutrophils, and the release of pro-inflammatory cytokines by peritoneal exudate cells were determined in the mice treated locally with Taurolin (TRD + PVP). The results were compared with control mice (placebo group), which received an equivalent dose of PVP. Local administration of Taurolin, in a dose relevant to a therapeutic dose used in the treatment of peritonitis in humans (10 mg TRD/kg body weight) (Baker *et al.,* 1994), completely inhibited zymosan-induced increased vascular permeability. As shown in Fig. 1A, Evans blue leakage from the circulation into the peritoneal cavity in TRD-treated mice was only slightly above the level of that in the untreated mice (spontaneous leakage). By contrast, taurine, as well as PVP, a stabilizer of TRD in Taurolin solution (Kirsh and Sihn, 1997), did not alter vascular permeability when compared to that of control mice (mice treated with zymosan only) (control OD = 0.360 ± 0.025).

Similarly, the influx of neutrophils into peritoneal cavity in zymosan-induced peritonitis, as measured by MPO activity, was completely blocked by TRD but not by taurine and PVP (Fig. 1B). In addition to beneficial effect of on vascular permeability and on neutrophil influx, TRD significantly attenuated inflammatory activities of peritoneal exudate cells by the reduction of TNF-α and IL-6 production. PVP and taurine did not affect the cytokine production (Fig. 1C).

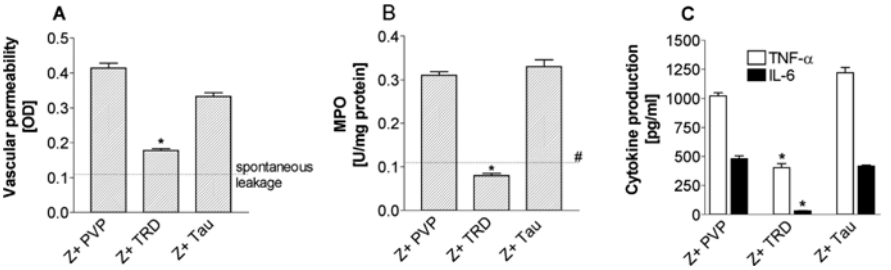


Figure 1. The effect of local TRD administration on the development of zymosan-induced peritonitis. PVP was used as placebo. * TRD *vs*. PVP p< 0.05. (A) Concentration of Evans blue in peritoneal exudates is shown as OD measured at 630 nm. The results represent 6 independent experiments. (B) MPO activity is expressed as units/1 mg of protein (results from 3 exp.) *MPO activity in peritoneal exudates of naïve, untreated mice. (C) TNF-α and IL-6 release from re-stimulated *in vitro* peritoneal exudate cells (results from 3 experiments).

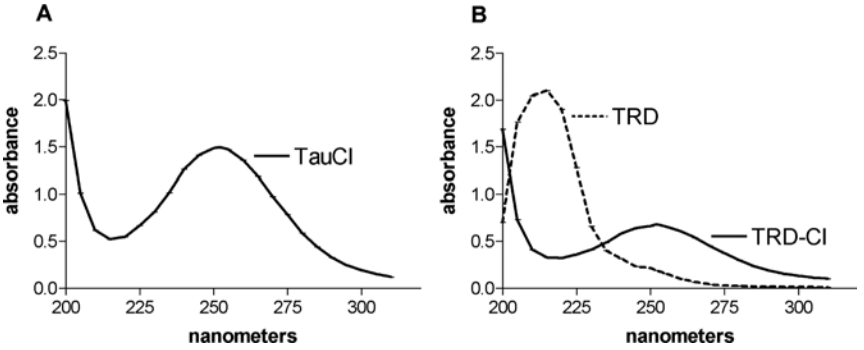


Figure 2. The UV absorption spectra of (A) TauCl (~3 mM), (B) TRD and TRD-Cl (~1.5 mM).

3.2. Effect of *in Vitro* HOCl Reaction With TRD

To determine whether TRD may react with HOCl to form monochloramine, similarly to the reaction between taurine and HOCl, the UV spectra of products of these reactions were analysed. As shown in Fig. 2, TRD and taurine react with HOCl in a similar way to form chloramines, as identified by characteristic UV spectra with λ_{max} for chloramines at 252 nm.

3.3 Effect of Taurine Derivatives on Macrophage Inflammatory Activities

3.3.1 TRD, TRD-Cl and TauCl Inhibit the Production of IL-6 by Activated Macrophages

TauCl, at non-cytotoxic concentrations, inhibited the production of IL-6 in both LPS- and IFN-γ stimulated macrophages in a dose-dependent manner. Taurine did not affect the production of IL-6.

TRD and its chlorinated derivative TRD-Cl inhibited the production of IL-6 by IFN-γ-stimulated macrophages in a similar dose-dependent manner (Fig. 3B). Surprisingly,

chlorination of TRD significantly enhanced its suppressive capacity as measured by the effect on IL-6 production by LPS-stimulated macrophages (Fig. 3A). In our experimental set-up TRD and TRD-CL were significantly stronger than TauCl in reducing IL-6 production.

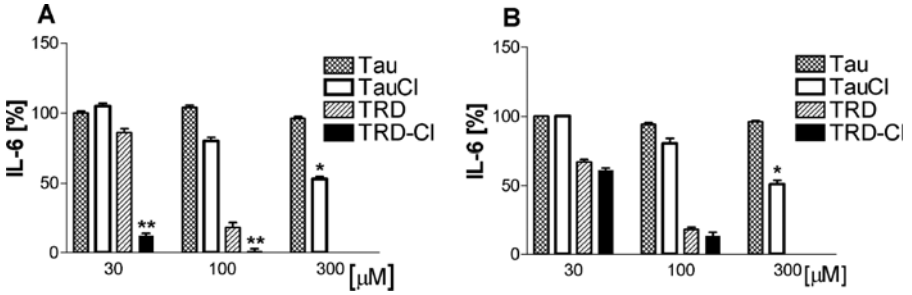


Figure 3. The effect of taurine derivatives on IL-6 release from peritoneal macrophages. Mϕ (5 x 10^5/ml) were stimulated either with LPS (A) or with IFN-γ (B) and cultured in the presence of Tau, TauCl, TRD or TRD-Cl. The agents were used at non-cytotoxic concentrations. Results represent 4 independent experiments. * Tau *vs.* TauCl $p < 0.05$; ** TRD *vs*. TRD-Cl $p < 0.01$.

3.3.2. TRD does Not Induce Expression of HO-1

TauCl induced the expression of HO-1 protein in non-stimulated macrophages in a dose-dependent manner. By contrast, TRD did not induce the expression of HO-1 in our experimental set-up (Fig. 4). Moreover, there was only a slight expression of HO-1 in Mø cytosol after incubation with taurine and with lower (30 μM) concentration of chlorinated TRD in some experiments.

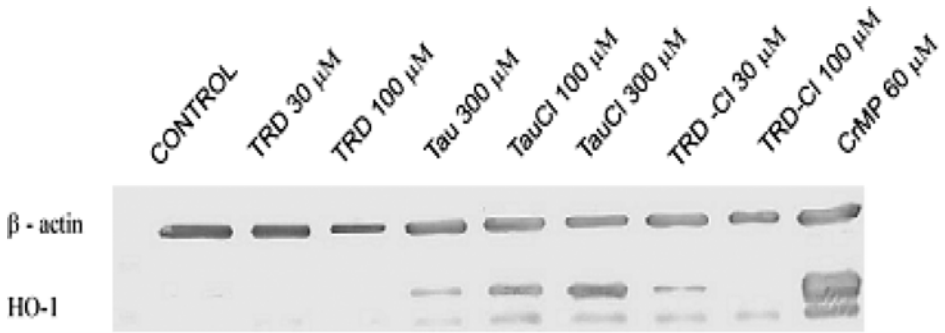


Figure 4. Representative Western blot of HO-1 protein expression in peritoneal macrophages 24 hours after stimulation with Tau, TauCl, TRD and TRD-Cl. Chromium III mesoporhyrin chloride (CrMP) was used as a reference inducer of HO-1 expression.

4. DISCUSSION

Taurolidine due to its anti-endotoxin, anti-bacterial and anti-adherence properties has been administered clinically by peritoneal lavage as a prophylaxis against infections after abdominal surgery and in the treatment of patients with established peritonitis (Browne *et al.,* 1976; Willatts *et al.,* 1995; Staubach, 1997). The bactericidal activity of TRD depends on the generation of active methylol groups. The methylol-containing moieties, taurultam and taurinamide (Scheme 1) appeared to react with bacterial cell wall components resulting in their denaturation (Myers and Allwood, 1980; Torres-Viera *et al.,* 2000). In addition to this direct effect on bacterial cell components, TRD has been reported to reduce the synthesis and activity of pro-inflammatory mediators probably by mechanisms independent of the methylol-containing species (Bedrosian *et al.,* 1991). Taurine, the third breakdown product of TRD, has been suggested to be responsible for its immunoregulatory properties (Watson *et al.,* 1995). Despite the lack of direct evidence, one may speculate that at a site of inflammation TRD may be chlorinated with HOCl to form chloramines, which will exert anti-inflammatory activities, as has been reported for TauCl (Marcinkiewicz, 1997; Schuller-Lewis and Park, 2003). These anti-inflammatory activities along with anti-bacterial and anti-endotoxin activities of TRD fully explain its beneficial therapeutic effect in acute peritonitis.

In our experimental model of acute peritonitis in mice we have shown that TRD exerts strong anti-inflammatory activities. Local administration of Taurolin, a chemioterapeutic, which contains Taurolidine and its stabilizer polyvinylpyrrolidine (PVP), reduced a number of parameters of acute inflammation induced by zymosan. Taurolin (TRD), but not PVP, reduced neutrophil infiltration into the peritoneal cavity. Moreover, plasmatic exudation, the effect of increased vascular permeability, was maintained by TRD at the level of spontaneous leakage. In addition, TRD significantly suppressed the production of TNF-α and IL-6, the two major pro-inflammatory cytokines, closely connected with the development of acute inflammatory states. All these results are in agreement with previous studies in which the effect of TRD on functions of inflammatory cells has been tested (Watson *et al.,* 1995). However, the link between the MPO-halide system and TRD has not been tested yet.

To evaluate the effect of MPO-halide system on the anti-inflammatory properties of TRD, we have tested *in vitro* the influence of TRD and that of chlorinated-TRD on macrophage functions. For the *in vitro* study, purified TRD, instead of Taurolin, was used and compared with TauCl as a reference taurine derivative. We have shown that TRD, as well as taurine, can react with HOCl to form chloramines. Whether, the formation of active chlorinated products of TRD, the most probably TauCl, affects the degradation of TRD into active methylol-moieties remains to be elucidated. Nevertheless our results indicate that *in vitro* at non-cytotoxic concentrations both TRD and TRD-Cl inhibit the production of IL-6 by LPS-stimulated macrophages. The effect of TRD was stronger when compared with that of TauCl which correlates with its stronger cytotoxic and pro-apoptotic activities (Table 1). Moreover, the production of other inflammatory mediators, such as NO and IL-12p40 was inhibited by TRD and TRD-Cl (data not shown). The suppressive effect was not dependent on the anti-endotoxin activity of TRD since the similar reduction of cytokine production was observed in macrophages stimulated with IFN-γ. Interestingly, chlorination of TRD, in contrast to chlorination of taurine, only slightly enhanced the suppressive activity of the parent molecule.

Table 1. Biological activities of TRD and taurine haloamines (TauCl and TauBr)

Activity	TRD	TauCl	TauBr
Cytotoxicity *in vitro* (Mϕ)	>100 μM	>300 μM	>300 μM
Antibacterial	yes	yes	yes
Induction of HO-1 expression	**no**	**yes**	**yes**
Antiendotoxin	yes	no	no
Pro-apoptotic activity	yes	yes	?
Inhibition of cytokine production (IC_{50})	~30 μM	~200 μM	~250 μM

While both native and chlorinated TRD exert anti-inflammatory activities, only taurine haloamines, but not native taurine, down-regulate the activity of inflammatory immune cells *in vitro* (Learn *et al.,* 1990; Marcinkiewicz *et al.,* 1995, 1999, 2005; Barua *et al.,* 2001). Although the precise mechanism of TauCl and TauBr action at a site of inflammation remains unclear, two scenarios, not excluding each other, have been proposed. Haloamines affect target immune cells either directly by inhibiting NFκB signalling, a pathway primarily involved in a cellular inflammatory response (Barua and Quinn, 2001; Kanayama *et al.,* 2002), or indirectly via the heme oxygenase system. Recently, we have described that TauCl and TauBr induce heme oxygenase-1 (HO-1) expression in resting and LPS-stimulated macrophages (Olszanecki and Marcinkiewicz, 2004). HO-1, a cytoprotective enzyme, is a part of the integrated response to oxidative stress. HO-1 catalyses heme degradation to iron, sequestered by ferritin, carbon monoxide (CO) and biliverdin (Abraham *et al.,* 1988; Vicente *et al.,* 2003). Several lines of evidence indicate a link between HO-1 and inflammation (Kushida *et al.,* 2002; Lee and Chau, 2002). We have shown previously that the induction of HO-1 expression by TauCl and TauBr is associated with a concomitant dose-dependent reduction of NO synthesis. As HO-1 products, as well as taurine haloamines, may suppress the production of pro-inflammatory mediators (Kushida *et al.,* 2002; Olszanecki and Marcinkiewicz, 2004), we may speculate that there is a link between taurine-dependent and HO-1-dependent cytoprotective mechanisms. Our present results indicate that TRD, in contrast to TauCl, at concentrations in which the reduction of cytokine production was observed, did not induce the expression of HO-1 protein. Thus, it is unlikely that the anti-inflammatory effect of TRD in zymosan-induced peritonitis is HO-1 dependent.

In conclusion, in this study we have shown TRD as an anti-inflammatory agent by mechanisms distinct from these responsible for immunoregulatory properties of TauCl and TauBr. The major difference was inability of effective dose of TRD to induce expression of HO-1 *in vitro*. Moreover, our results do not confirm the suggestion of Watson *et al.* (1995) that TRD functions primarily through the taurine moiety. However, at a site of inflammation, taurine, as a breakdown product of TRD, may react with HOCl to produce anti-inflammatory agent, TauCl. Further studies are necessary to determine the influence of TRD chlorination on both formation and biological activity of all three TRD breakdown products, taurinamide, taurultam and taurine.

5. SUMMARY

In this study, in an animal model of zymosan-induced peritonitis we have tested anti-inflammatory properties of Taurolidine (TRD), a synthetic derivative of taurine. *In vitro,*

the effect of TRD and HOCl treated TRD on peritoneal macrophages was compared with that of TauCl. We report that locally administered TRD (Taurolin) shows strong anti-inflammatory properties. TRD inhibits vascular permeability increased by inflammatory stimuli; it also significantly attenuates the influx of neutrophils into the peritoneal cavity, as well as the production of pro-inflammatory cytokines (TNF-α, IL-6) by peritoneal exudate cells. Chlorination of TRD resulted in the formation of chloramine (TRD-Cl), as confirmed by characteristic UV spectra. Both TRD and TRD-Cl, more effectively than TauCl, inhibited the production of IL-6 by stimulated macrophages. The effect was not dependent on its well-known anti-endotoxin activity since TRD inhibited cytokine production by macrophages stimulated with either LPS or IFN-γ.

Finally, we report that anti-inflammatory activities of TRD and taurine haloamines are mediated by different mechanisms. TRD, in contrast to TauCl and TauBr, does not induce expression of HO-1, a stress inducible enzyme with strong anti-inflammatory properties.

6. ACKNOWLEDGMENTS

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TAURINE CHLORAMINE INHIBITS THE PRODUCTION OF NITRIC OXIDE AND SUPEROXIDE ANION BY MODULATING SPECIFIC MITOGEN-ACTIVATED PROTEIN KINASES

Chaekyun Kim*, Hyung Sim Choi, and Jun Woo Kim

1. INTRODUCTION

Taurine is an abundant free amino acid in inflammatory cells (Fukuda and Usui, 1983; Vinton *et al*., 1986), and it has been claimed to protect cells from inflammatory injury. Taurine readily reacts with hypochlorous acid ($HOCl/OCl^-$) produced by the myeloperoxidase system in neutrophils (Thomas *et al*., 1985) and forms more stable and less toxic taurine chloramine (Tau-Cl). Tau-Cl protects inflammatory cells by attenuating the toxicity of $HOCl/OCl^-$. Tau-Cl also protects cells by regulating the production of many pro-inflammatory mediators, such as nitric oxide (NO), superoxide anion (O_2^-), tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-8 and prostaglandin (Park *et al*., 1995; Kim *et al*., 1996; Marcinkiewicz *et al*., 1998).

Mitogen-activated protein kinases (MAPK), which include extracellular signal-regulated kinase (ERK), p38 and c-Jun N-terminal kinase, regulate many cellular functions including production of pro-inflammatory mediators. In particular, MAPK regulate signaling pathways involved in NO and O_2^- production (Chan and Riches, 2001; Kim and Dinauer, 2001). It is not much known about the role of taurine on MAPK activation. Midwinter *et al*., (2004) reported that Tau-Cl alone induced ERK activation in human vein endothelial cells. However, Tau-Cl showed no effect on ERK activation in Jurkat cells (Kontny *et al*., 2003). In addition, the activation of MAPK leads to activation of transcription factors (Angel and Karin, 1991; Baeuerle and Baichwal, 1997) which are strongly involved in the expression of pro-inflammatory genes such as nuclear transcription factor (NF-κB) and activator protein 1 (AP-1). Recently, it has been shown that Tau-Cl inhibits the activation of NF-κB in inflammatory cells (Barua *et al*., 2001; Kanayama *et al*., 2002).

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In this study, we investigated the effect of Tau-Cl on the production of NO and O_2^-, and the activation of MAPK (ERK and p38) that lead to NO and O_2^- production. In consistent with previous results, Tau-Cl inhibited NO and O_2^- production in a dose-related manner. Tau-Cl inhibited LPS-stimulated phosphorylation of ERK1/2 in RAW 264.7 cells, while it had no effect on p38. However, Tau-Cl inhibited PMA-elicited phosphorylation of p38 in PLB 985 cells but not ERKs. These results suggest that Tau-Cl regulates pro-inflammatory mediators in a stimulus- and signaling-pathway-specific manner.

2. MATERIALS AND METHODS

2.1. Antibodies and Reagents

Rabbit polyclonal antibodies against ERK1/2, phospho-ERK1/2, and phospho-p38 were purchased from New England Biolabs (Beverly, MA). Chemicals were purchased from Sigma (St. Louis, MO) unless otherwise stated. Fetal bovine serum (FBS) was from HyClone (Logan, UT), and penicillin, streptomycin, and HBSS were from GibcoBRL (Grand Island, NY). Tau-Cl was freshly synthesized on the day of use by adding equimolar amounts of NaOCl (Aldrich Chemical, Milwaukee, MI) to taurine. The authenticity of Tau-Cl formation was monitored by UV absorption (200-400 nm) (Thomas *et al*., 1986). Endotoxin-free or low endotoxin grade water and buffers were used.

2.2. Cell Culture and Murine Peritoneal Neutrophils

Murine macrophage cell line, RAW 264.7 cells (ATCC, Manassas, VA) were grown in DMEM supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin and 10% FBS at 37ºC in 5% CO_2. The human myeloid cell line PLB-985 was a gift from M. Dinauer (Indiana University). PLB-985 cells were grown in RPMI 1640 medium containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37ºC in 5% CO_2. To induce granulocytic differentiation, PLB-985 cells were exposed to 0.5% *N,N*-dimethylformamide for 5 days.

To elicit peritoneal neutrophils, C57BL6/J mice (Jackson Lab, Bar Harbor, MA) were injected intraperitoneally with 1 ml of 3% thioglycollate. Peritoneal cells were harvested with HBSS after 18 h as previously described (Kim *et al*., 1996).

2.3. Measurement of Nitric Oxide

Nitrite, a stable end product of NO present in the conditioned media, was determined by Griess reaction. Briefly, the conditioned media (100 μl) from RAW 264.7 cells stimulated with LPS (1 μg/ml) for 20 h were reacted with an equal volume of Griess for 10 min at room temperature. The absorbance was measured at 550 nm using a Power Wavex 340 ELISA reader (Bio-Tek instruments, Winoosk, VT).

2.4. Cytochrome c Reduction Assay

Extracellular O_2^- production was measured based on superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome c (Kim and Dinauer, 2001). Briefly, 2.5 x 10^5 cells were suspended in 250 μl PBSG (PBS with 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 7.5 mM glucose) containing 75 μM ferricytochrome c and activated by the addition of 200 ng/ml PMA. 180 units/ml SOD was added to parallel samples to measure the SOD-inhibitable values. After incubation for 30 min at 37ºC, the absorbance at 550 nm with a 490 nm reference filter was measured on a Power Wavex 340 ELISA reader.

2.5. MAPK Activation and Western Blotting

To examine the extent of the phosphorylation of MAPK, cells (1 x 10^7) treated with taurine or Tau-Cl were lysed in 200 μl of lysis buffer containing 20 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 20 μg/ml chymostatin, 2 mM PMSF, 10 μM leupeptin and 1 mM 4-(2-aminoethyl)benzenesulfonylfluoride, 50 mM NaF and 2 mM Na_3VO_4 for 30 min at 4ºC. Cell lysates were clarified by centrifuging at 18000 x g for 2 min at 4ºC. Protein was quantified using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Thereafter, samples (10 μg) were heated with 5X Laemmli sample buffer for 5 min at 95ºC and then resolved on 10% SDS-PAGE. Separated proteins were transferred onto nitrocellulose membrane (MSI, Westborough, MA), and the non-specific bindings were blocked with 6% non-fat milk dissolved in TBST buffer (10 mM Tri-Cl, pH 8.0, 150 mM NaCl, 0.05% Tween 20). Phosphorylation was detected using phospho-specific ERK1/2 and p38 antibodies and the total contents of ERK1/2 were detected using antibodies against ERK1/2.

2.6. Statistical Analysis

The two-tailed Student's t-test (paired) was performed using Microsoft Excel software (Redmond, WA). Data are expressed as mean ± SD and a p value < 0.05 was considered significant.

3. RESULTS AND DISCUSSION

3.1. Tau-Cl Inhibited LPS-Induced NO Production in RAW 264.7 Cells

It has been previously shown that Tau-Cl inhibits the LPS and interferon (IFN)-γ-dependent production of pro-inflammatory mediators in murine macrophages. In particular, the inhibitory effect of Tau-Cl on NO production was intensively studied, and possible mechanisms were suggested (Park *et al*., 1993, 1995; Marcinkiewicz *et al*., 1995; Barua *et al*., 2001). In this study, LPS (1 μg/ml) enhanced the NO production in RAW 264.7 cells, and LPS-induced NO production was inhibited by Tau-Cl in a dose-dependent manner (Fig. 1A). However, taurine did not show significant inhibitory effect. The concentrations used in this study (0.5 and 1 mM) did not cause significant non-specific cell death when measured by trypan blue exclusion or MTT (thiazolyl blue tetrazolium bromide) assay (Fig. 1B).

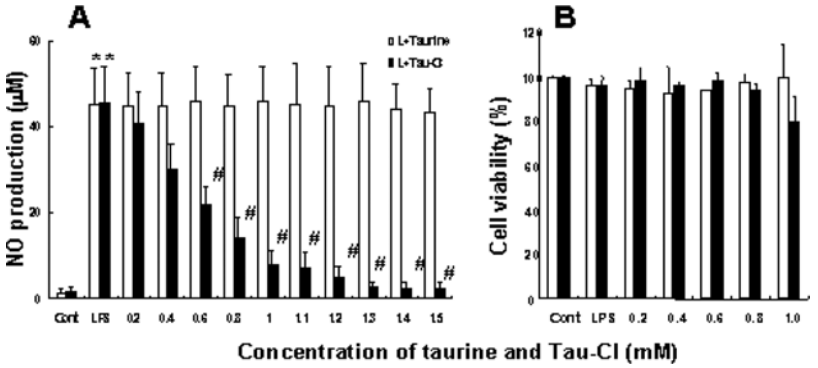

Figure 1. Tau-Cl inhibits LPS-induced NO production in macrophages. *A.* RAW 264.7 cells were treated with taurine or Tau-Cl in the presence of LPS, NO production was measured by Griess reaction (n=4). Data were expressed as mean values ± SD, * $p<0.05$, control *vs.* LPS, # $p<0.05$, LPS *vs.* Tau-Cl. *B.* Cell viability after treatment of taurine and Tau-Cl was measured by MTT assay (n=5).

3.2. Tau-Cl Inhibited PMA-Elicited O_2^- Production in PLB-985 Cells

We previously reported that that Tau-Cl inhibits the PMA-elicited O_2^- production in murine peritoneal neutrophils (Kim *et al*., 1996). In this study, we examined the effect of Tau-Cl on O_2^- production in PLB-985 cells which differentiated to granulocytes. Tau-Cl inhibited PMA-elicited O_2^- production, while taurine did not have any significant inhibitory effect (Fig. 2A). This result does not agree with our previous result which showed that taurine inhibited O_2^- production in murine peritoneal neutrophils (Kim *et al*., 1996). Thus, we measured O_2^- production using murine peritoneal neutophils. In murine neutophils, Tau-Cl inhibited O_2^- production similarly as in PLB-985 granulocytes, while taurine slightly inhibited O_2^- production (Fig. 2B). These combined results suggest that

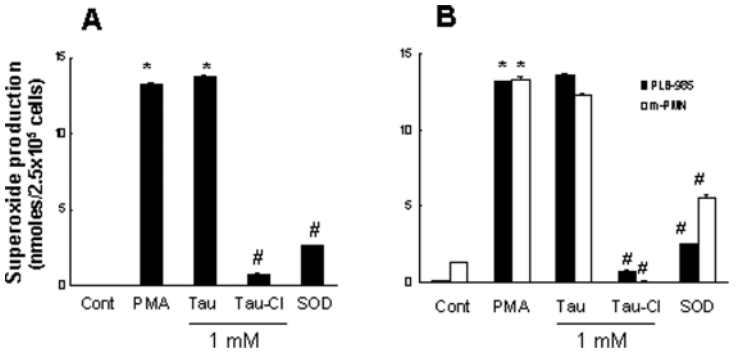

Figure 2. Tau-Cl inhibits PMA-elicited superoxide anion production in neutrophils. (A) Superoxide production following the stimulation with 200 ng/ml PMA in PLB-985 granulocytes was monitored by the reduction of cytochrome c (n=5). (B) Comparison of superoxide production between PLB-985 granulocytes and murine peritoneal neutrophils (n=5). Data were expressed as mean values ± SD, * $p<0.05$, control *vs.* PMA, # $p<0.05$, PMA *vs.* Tau-Cl or SOD.

the inhibition of NO and O_2^- production by Tau-Cl protects cells from inflammatory injury caused by overproduction of reactive nitrogen/oxygen species.

3.3. Tau-Cl Selectively Inhibited MAPK Activation

Although it is controversial, the involvement of ERK and p38 in LPS-stimulated NO production or PMA-stimulated O_2^- production has been reported (Bhat *et al*., 1998; Ajizian *et al*., 1999; Karlsson *et al*., 2000; Kim and Dinauer, 2001; Watters *et al*., 2002). As a possible mechanism which Tau-Cl inhibits NO and O_2^- production, we hypothesized that Tau-Cl may interfere with MAPK signaling pathway. We examined the effect of Tau-Cl on either LPS- or PMA-induced phosphorylation of ERK and p38. Tau-Cl inhibited LPS-stimulated phosphorylation of ERK1/2 in RAW 264.7 cells without affecting the phosphorylation of p38 (Fig. 3A). However, Tau-Cl did not inhibit PMA-stimulated phosphorylation of ERK in PLB-985 cells (Fig. 3B).

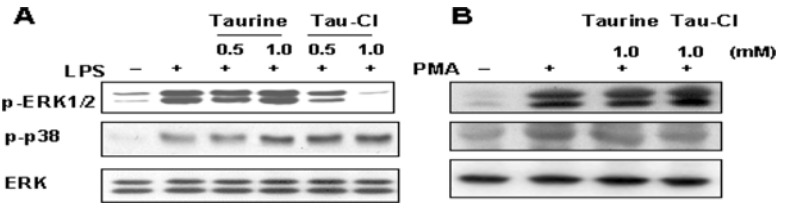

Figure 3. Tau-Cl inhibits the activation of specific MAPK. Cells (1 x 10^7) were pretreated with taurine and Tau-Cl for 20 min before stimulation with LPS (1 μg/ml) for 15 min. Cells were lysed and western blotting was performed with the antibodies for phospho-ERK1/2 or phospho-p38, and same blot was reprobed with ERK1/2 antibody (n=5).

In summary, we show that Tau-Cl inhibits LPS-induced NO production by inhibiting ERK phosphorylation and PMA-stimulated O_2^- production by inhibiting p38 phosphorylation, suggesting stimulus-specific regulation of reactive nitrogen/oxygen species by Tau-Cl.

4. ACKNOWLEDGMENTS

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ANTI-NEUROTOXIC EFFECTS OF TAUROPYRONE, A TAURINE ANALOGUE

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1. INTRODUCTION

The novel type of amino acid-containing 1,4-dihydropyridine (DHP) analogues were synthesized at the Latvian Institute of Organic Synthesis, Riga, Latvia, that have lead to the conversion of calcium antagonistic properties (typical for classical DHPs) to non-calcium antagonistic ones (for novel compounds). Besides, novel compounds have become highly soluble in comparison to typical DHP compounds, such as nifedipine and nimodipine, which are insoluble in water. Moreover, amino acid-containing DHPs can be regarded as unnatural dipeptides since their structures resemble peptidomimetic substances: the peptide bound (-CONH-) joins two amino acid elements: free and "crypto" ones. The free amino acid moiety is, for instance, the glutamate, taurine or gamma-aminobutyric acid (GABA) residue, attached to the position 4 of the DHP ring, whereas the so-called "crypto" amino acid elements are included into the DHP ring as unsaturated amino acid moieties (Fig. 1). DHP ring is considered as essential carrier moiety for the better delivery of such type of molecules to the brain.

We have found previously that the peptidomimetic structure of novel compounds exerted typical for peptides effects. Thus, mostly studied glutapyrone and gammapyrone (glutamate- and GABA-containing DHP analogues, respectively) showed that these compounds acted at low doses (mostly micrograms) in *in vivo*, showed very low toxicity (e.g. for glutapyrone LD_{50} >5000 mg/kg i.p., vs classical DHP, 200-600 mg/kg**),** and demonstrated typical for peptides neuroregulatory (influence on classical neurotransmitter biosynthesis, release and metabolism) and regulatory repertoire of their effects. Thus, glutapyrone showed antidepressant (Misane *et al.,* 1998), anticonvulsant **(**Karpova *et al.,*

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Taurine 6
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activities in intact and alcoholised rat offspring (Klusa and Germane, 1996). In *in vitro* 1993*)* stress-protective (Tarasenko *et al.,* 2002), neuroprotective/memory-stimulating

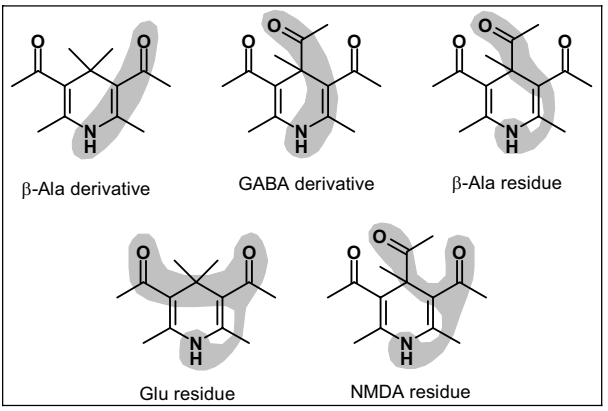


Figure 1. „Crypto" amino acid moieties (grey areas) involved into the dihydropyridine ring.

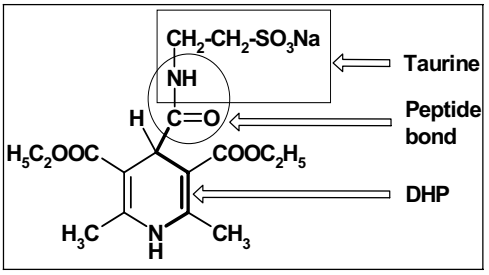


Figure 2. Structure of tauropyrone.

experiments glutapyrone and gammapyrone stimulated protein kinase C (PKC) activity in cultured aortic smooth-muscle cells A7r5 (Kluša *et al.,* 1996), glutapyrone was capable to protect the azidothymidine (AZT)-induced mitochondria aggregation (Velena *et al.,* 1997).

Fig. 2 shows tauropyrone, a taurine-containing DHP, designed as dipeptide-like structure, comprising both the free amino acid residue of taurine, and „crypto" amino acid moiety – GABA (in bold). Tauropyrone is less studied compound among synthesized DHP analogues of amino acid-containing DHP series. Previously it was found that tauropyrone possesses anti-aggregant properties in human blood platelet assay (Poikans *et al.,* 1994**).** Preliminary data obtained in *in vivo* experiments in rats showed markedly expressed alcohol intake-reducing effect of tauropyrone, and it is more prolonged than that of taurine restorative influence on memory processing in alcohol-neurodeficiency rats (Klusa *et al.,* 1997).

The present study is the first attempt to clarify cellular targets of tauropyrone action in *in vitro* tests. Taking into account data on taurine as active neuroprotector in different neuronal injury conditions (for review see: Della Corte *et al.,* 2002), we consider that tauropyrone will also be capable to act as a putative anti-neurotoxic agent. We have examined here tauropyrone in several neurotoxicity models in cerebellar granule cells. For comparison, cerebrocrast as the reference compound with classical DHP structure (comprising both phenyl and DHP rings) was used, since this compound was previously found to be a highly active neuroprotective (Klusa, 1995) and anti-inflammatory agent (Klegeris *et al.,* 2002).

2. MATERIALS AND METHODS

2.1. Chemicals

DNAse I (from bovine pancreas, lypophilized, sterile-filtered), trypsin (from bovine pancreas, salt free, non-sterile), and trypsin inhibitor were from Boeringer Manheim GmbH (Manheim, Germany); L-glutamine, cytosine arabinofuranoside, poly-L-lysine, foetal bovine serum from Sigma Chemical Co. (St. Louis, MO, USA); and Eagle's basal medium (BME) with Earle's salts from GIBCO BRL (Roskilde, Denmark). Tauropyrone [2-(2,6-dimethyl-3,5-diethoxycarbonyl-1,4-dihydropyridine-4-carboxamido)ethansulpho acid], and the reference drug cerebrocrast [4-2(difluoromethoxy)phenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylic acid di(2'-proepoxyethyl)diester] were synthesized at the Latvian Institute of Organic Synthesis, Riga, Latvia.

2.2. Cerebellar Granule Cell Cultures

Primary cultures of cerebellar granule cells were prepared according to the method described by Gallo *et al*. (1982) with minor modifications. In brief, cells were obtained from 8-day old rats, incubated in 0.025% trypsin and dispersed by trituration in a 0.004% DNAse and 0.05% trypsine inhibitor-containing solution. Cerebellar granule cells were resuspended in Basal Medium Eagle with Earle's salts (BME) supplemented with 10% foetal bovine serum, 25 mM KCl, 2 mM glutamine, 100 ng/ml gentamycin and seeded at the density of 1.4×10^6 cells/ml on 35 mm culture dishes pre-coated with poly-L-lysine (5 μg/ml). Cytosine arabinofuranoside (10 μM) was added 24 h after plating to prevent the proliferation of non-neuronal cells. Cells were cultured for 8 days in an atmosphere of 5% CO_2/95% air at 37°C.

2.3. Oxygen-Glucose Deprivation Model

2.3.1. Assessment of Neuroprotective Activity Against Oxygen-Glucose Deprivation

Oxygen-glucose deprivation (OGD) was induced according to the method described by Goldberg and Choi (1993) with minor modifications. Cerebellar granule cells were grown for 8 days, and for OGD induction the culture media were replaced by a through exchange with a deoxygenated, glucose-free balanced salt solution (mM): NaCl 116, KCl 5.4, $MgSO_4$ 0.8, NaH_2PO_4 1, $CaCl_2$ 0.9 and phenol red 10 mg/l. The cultures were put into an anaerobic chamber (Forma Scientific, USA) containing a gas mixture of 85% N_2,

10% H_2 in 5% CO_2 at 37°C. Duration of OGD was 90 min to induce moderate intensity OGD or 100 min for strong intensity OGD. Cell damage was assessed by the measurement of lactate dehydrogenase (LDH) activity using LDH kit. The decrease in absorbency at 340 nm during 3 min at 30 s interval was determined using spectrophotometer. LDH content (U/ml) was calculated from the slope of linear portion of the absorbency curve.

2.4. Glutamate Toxicity Model

2.4.1. Assessment of Cell Viability

Test compounds were added during glutamate (20 μM) exposure (30 min) and remained in the medium for 20 h until cell viability toxicity was assayed by 3-(4,5-dimethyl-thiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) quantitative colorimetric method (Mosmann, 1983).

2.4.2. Measurement of Production of Free Radicals

Test compounds were added during glutamate (500 μM) exposure (60 min). The formation of intracellular free radicals was measured using 2',7'-dichlorofluoresceine diacetate (DCF-DA, 50 μM), which was added to the cells 20 min after glutamate for 40 min. Drugs were added together with glutamate. Thereafter, the cells were washed with Locke's buffer (154 mM NaCl, 5.6 mM KCl, 2.3 mM $CaCl_2$, 3.6 mM $NaHCO_3$, 5.5 mM D-Glucose, 5 mM HEPES, pH=7.5). Cell fluorescence images were obtained using laser confocal microscope MRC 1024 ES (Bio-Rad). The percentage of cells with increased free radical production (fluorescence intensity more than 200 pixels) was expressed as percentage from total number of cells counted. At least 2 dishes for one drug dose were used in each experiment and 5 images from each culture dish were randomly taken and the average values calculated. The obtained data were verified in at least two independent experiments.

2.5. MPP^+ Model

2.5.1. Measurement of Formation of Free Radicals

The oxidative stress was induced by addition of MPP^+ (300 μM) for 60 min. The formation of intracellular free radicals was measured using DCF-DA (50 μM), which was added to the cells 20 min after MPP^+ for 40 min. Assessment of free radical formation was similar to that in case of glutamate toxicity test.

2.6. Statistics

Data were calculated as the mean ± SEM. One-way analysis of variance (ANOVA), followed by Bonferroni multiple comparisons test was performed to statistically differentiate mean values among groups.

3. RESULTS

Fig. 3 shows that oxygen-glucose deprivation induced considerable cell damages of cerebellar granule cells that have led to markedly increased activity of lactate dehydrogenase (LDH). Tauropyrone added to the culture of cerebellar granule cells was capable to protect cells from OGD-induced damages. The influence of tauropyrone on LDH activity depended on the OGD intensity: at moderate OGD (LDH release by 73% in comparison to control), the protective effect of tauropyrone at 10 μM was about 80% (Fig. 3A), whereas at strong intensity OGD (LDH release by 140% *vs*. control), tauropyrone protective ability at 10 and 100 μM was only about 37% (Fig. 3B). Cerebrocrast showed a pronounced anti-neurotoxic activity (protection by about 85%) already at 0.1 μM, while less activity was observed at 1 μM, and it was inactive at 10 μM in moderate OGD intensity experiment (Fig. 3A). In case of strong OGD intensity, cerebrocrast was slightly active (comparable to tauropyrone) at 0.01 and 0.1 μM, and ineffective at 1 μM (Fig. 3B).

In glutamate (20 μM, exposure 30 min, assessment in 20 h) toxicity test (MTT assay), cell viability was reduced to 40% *vs*. non-glutamate control (100%). Tauropyrone at concentrations 1, 10 and 100 μM protected cell death caused by glutamate: cell viability was observed as 60-70% *vs*. glutamate control (40%). There was no markedly expressed concentration-effect relationship (Fig. 4). Cerebrocrast (0.1 μM) was ineffective in this test.

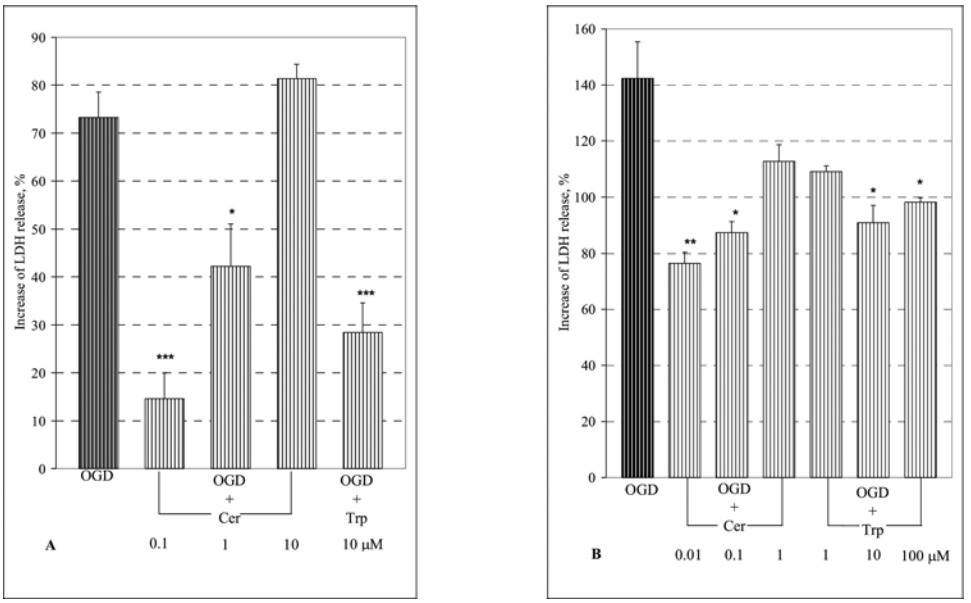


Figure 3. The protective effect of tauropyrone (Trp) and cerebrocrast (Cer) against the oxygen-glucose deprivation (OGD) cell damages in moderate intensity (A) and strong intensity (B) increased LDH release in cerebellar granule cells. Drugs were added during and after OGD. Release of LDH in control culture was taken as 0%. * $p<0.05$ *vs.* OGD, ** $p<0.01$ *vs.* OGD, *** $p<0.001$ *vs.* OGD; one way ANOVA, followed by Bonferroni multiple comparisons test.

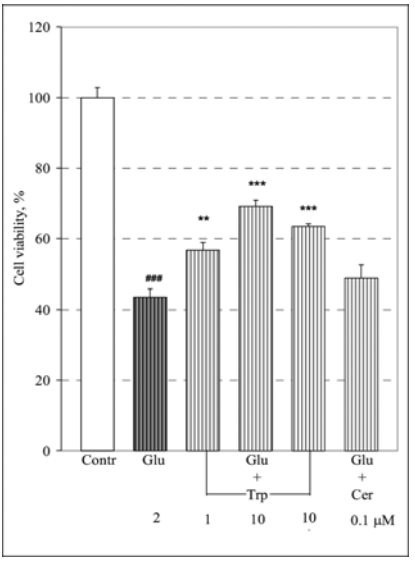
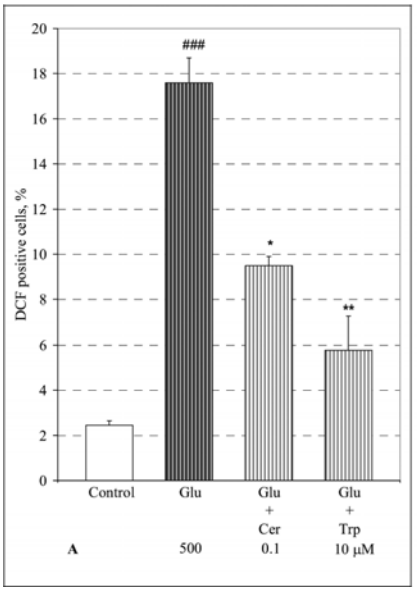


Figure 4. The influence of tauropyrone (Trp) and cerebrocrast (Cer) on the cell death induced by glutamate (Glu) in cerebellar granule cells. Cells were exposed to glutamate (20 μM) for 30 min. Drugs were added during and after glutamate exposure for 24 h. Cell viability was measured by MTT assay. ### p<0.001 *vs.* control; ** p<0.01 *vs.* Glu; *** p<0.001 *vs*. Glu; one way ANOVA, followed by Bonferroni multiple comparisons test.



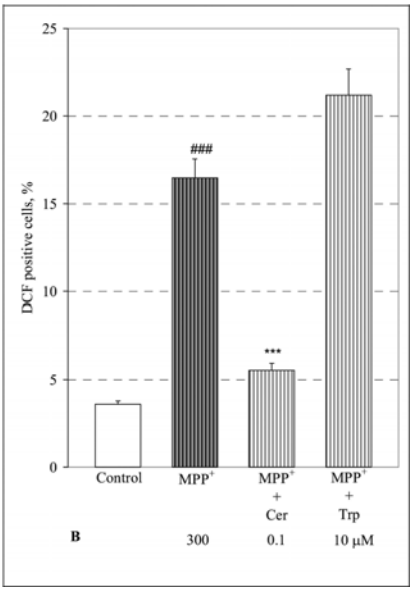


Figure 5. The effects of tauropyrone (Trp, 10 μM) and cerebrocrast (Cer, 0.1 μM) on the glutamate (Glu)-induced (A) and MPP^+-induced (B) production of free radicals (FR) in cerebellar granule cells. Glu, 500 μM; MPP^+, 300 μM. ### p<0.001 *vs.* control; * p<0.05 *vs.* Glu; ** p<0.01 *vs.* Glu, *** p<0.001 *vs.* MPP^+; one-way ANOVA followed by Bonferroni multiple comparisons test.

When tauropyrone effect on free radical formation was tested, glutamate toxicity was caused by its concentration of 500 μM (exposure 60 min followed by immediate assay). In comparison to control, glutamate-induced free radical production by 80% in average vs control. Tauropyrone at concentration 10 μM considerably (more than 2-fold) reduced glutamate-induced free radical formation. The action of tauropyrone was even more expressed than that of cerebrocrast (Fig. 5A).

The data presented in Fig. 5B show that tauropyrone (10 μM) did not affect MPP^+-induced free radical formation measured as a number of DCF positive cells, whereas cerebrocrast at 0.1 μM almost completely reduced MPP^+-induced neurotoxicity.

4. DISCUSSION

We have tested tauropyrone, a novel tauropyrone-containing DHP analogue, in oxygen-glucose deprivation (OGD), glutamate and MPP^+ *in vitro* models. The obtained data are the first ones that characterize an ability of tauropyrone to act as a putative anti-neurotoxic agent.

OGD is a severe injury to brain tissue when loss of oxygen (ischemia) and glucose delivery leads to cell damage by different pathways. They include energy failure, acidosis, glutamate excitotoxicity, calcium mediated toxicity, oxidative stress (Kristian and Siesjö, 1998; Fujimoto *et al.,* 2004). During cerebral ischemia, the overactivation of glutamate receptors, calcium channels and sodium channels leads to excitotoxic neuronal injury through enhancing glutamate efflux into the extracellular space (Kimura *et al.,* 1998; Velly *et al.,* 2003). Ischemia induces the impairment of mitochondrial activity and the consequent overproduction of free radicals in neurons (Scorziello *et al.,* 2001). Particularly sensitive to OGD is the neurotransmission, which is rapidly impaired and, when the duration of the ischemic episode lasts for more than a few minutes, the disappearance of synaptic responses becomes irreversible. Thus, disappearance of synaptic transmission is considered as the first detectable functional sign of cell suffering that eventually results in neuron death (Pedata *et al.,* 1993).

Results of present studies have shown that tauropyrone was capable to protect the OGD-induced cell damage in cerebellar granule cell culture. The protective ability (reduction of OGD-induced LDH release) was dependent on the intensity of OGD toxicity (moderate intensity was defined as LDH release of 73%, strong intensity of 140%). At moderate OGD intensity, the protective effect of tauropyrone at 10 μM was about 80% (Fig. 3A), whereas at strong OGD intensity tauropyrone effect at 10 and 100 μM was only 37% (Fig. 3B). Cerebrocrast, a reference drug with classical DHP structure that comprises two cyclic moieties (phenyl cycle and DHP ring), was strongly active at 0.1 μM, less active at 1 μM and inactive at 10 μM in the moderate OGD intensity experiment; and slightly active (comparable to tauropyrone) at 0.01 and 0.1 μM, and ineffective at 1 μM in strong intensity OGD (Fig. 3). This experiment also showed that there was no strictly expressed concentration-effect relationship either in tauropyrone or cerebrocrast cases. Mostly after reaching the maximum effect, a *plateau* effect or even inverse effect can be observed by increasing concentrations. Similar phenomenon was demonstrated also in taurine studies where higher neuroprotective activity was observed at lower (2 mM) than at higher (20 mM) concentration in hippocampal slices by use of MPP+ toxicity model (O'Byrne *et al.,* 2000).

Although at present it is difficult to define which cellular target is the most essential in preventive action of tauropyrone in OGD model, involvement of its influence on glutamatergic system can be taken as essential, since tauropyrone showed anti-neurotoxic effects also in glutamate-toxicity model. Both models OGD and glutamate ones can be attributed to loss of cellular homeostasis and overload of glutamate, a phenomenon termed glutamate excitotoxicity. In glutamate-toxicity model (glutamate concentration 20 μM), tauropyrone (1, 10 and 100 μM) significantly but not in concentration-dependent manner protected cell death (Fig. 4). However this effect can be evaluated as slightly expressed. At the same time cerebrocrast at concentration 0.1 μM that has been revealed as the most active concentration in our previous and also in these studies, was ineffective. Tauropyrone was highly active in protection of glutamate (500 μM)-induced free radical production (Fig. 5A). At concentration 10 μM its activity was more expressed (protection by 65% in average) than that of cerebrocrast at 0.1 μM (protection by 45% in average). Therefore on may say that tauropyrone protective action involves glutamatergic component probably by regulating the OGD excitotoxicity-released toxic glutamate. We have not yet obtained the data about ability of tauropyrone to bind directly to NMDA or non-NMDA, or to matabotropic glutamate receptors receptors, which role in calcium-influx-induced necrotic cell damage (Yoshioka *et al.,* 2000) and OGD-induced neuronal apoptosis (Kalda and Zharkovsky, 1999) is well-established. However, judging from inability of other amino acid-containing DHP analogues, such as glutapyrone and gammapyrone (glutamate- and GABA-containing, respectively) to bind to glutamate receptor subtypes (Misane *et al*., 1998), one may expect this inability also for tauropyrone. Moreover, we speculate that indirect peptide-like modulatory effect of glutamatergic processes can be taken as crucial mechanism of tauropyrone action.

Unlike effectiveness of tauropyrone in OGD and glutamate toxicity models, our experiments in MPP^+ model have not revealed a neuroprotective effect of tauropyrone (Fig. 5B). MPP^+, the active cytotoxic metabolite of 1-methyl-4-phenyl-1,2,3,6-tetra-hydropiridine (MPTP), is being incorporated into cerebellar granule cells via both cationic transporter and dopamine transporter, causes intracellular free radical generation and cell death by interfering with mitochondrial bioenergetics at the level of complex I which deficiency is considered as critical cause for Parkinson disease etiopathology. One may consider that mitochondrial complex I is not a crucial target involved in tauropyrone protective effects, since it did not influence the MPP^+-induced neurotoxicity. At the same time cerebrocrast was found as highly active neuroprotector against MPP^+-induced damages. However, other mitochondrial targets of tauropyrone action cannot be excluded, and its ability to protect cell loss caused by glutamate may be focused on at present unknown mitochondrial pathways. These data showed diversity in mechanisms of amino-acid-containing DHP and classical DHP compounds. Other experiments in liver mitochondria (Fernandes *et al.,* 2003) confirms these suggestions by demonstrating that only cerebrocrast but not amino acid-containing DHP compounds, such as glutapyrone and gammapyrone, acted as regulators of mitochondrial processes, namely at the level of inner mitochondrial anion channels, K^+/H^+ antiporter and Ca^{2+} -induced mitochondrial permeability transition pore. It can be noted that ability of taurine to protect MPP^+ toxicity in coronal slices from rat brain was detected in high millimolar concentrations, mostly at 1 and 20 mM (Della Corte *et al.,* 2002). We have used in MPP^+ model micromolar concentrations at which highly effective protection was found in both OGD and glutamate models.

Summarizing the data obtained in the present studies, one may suggest that the tauropyrone structure is more suitable to protect neuronal cells against OGD-induced hypoxic/ischemic injury, most likely by alleviating disturbances of OGD excitotoxicity-released toxic glutamate, or modulatory action at the level of glutamatergic system. This cellular target can be considered as useful to stop early stages of the pathogenic cascade leading to neurodegeneration particularly that induced by brain hypoxia/ischemia (stroke). Further studies may give beneficial results in understanding tauropyrone effects and its usefulness as the therapeutic agent.

5. ACKNOWLEDGMENTS

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TAURINAMIDE DERIVATIVES – DRUGS WITH THE METABOLIC TYPE OF ACTION

Minireview

Nikolay S. Sapronov and Ludmila K. Gavrovskaya*

1. INTRODUCTION

In the last years the concept on the role of oxidative stress in disease pathogenesis of both central nervous system (CNS) and visceral system was generally recognized. Generation of active oxygen forms, regulation disorders of antiradical protective mechanisms, exhaustion of the endogenous antioxidant system, and incontrollable activation of lipid peroxidation (LP) play a crucial part in cell death. In some clinical and biological studies, intensification of pathogenic LP processes and decrease in antioxidant protection was shown in patients with vascular diseases of different organs. At the same time, standard therapy was ineffective in normalization of LP activity and did not promote the increase of the endogenous antioxidant background. Considering the importance of the oxidative stress in many pathological states, it is necessary search for drugs with the metabolic type of activity, having antioxidant, antihypoxic, anti-ischemic, membrane-stabilizing and reparative activity for treating diseases caused by hypoxic and ischemic disorders.

Due to its polyactivity taurine is known to play an important role in regulation of metabolic processes (Huxtable, 1992; Saransaari and Oja, 2000). Interest to taurine is primarily the result of its membrane-stabilizing influence on cell membranes; neuromodulation in the CNS; neurotransmission in the specialized systems; regulating effects on metabolic processes (energy, carbohydrate, protein); influence on the body endocrine functions and production of paired bile acids (Gurevich, 1986; Nefedov, 1992). Complexity of the pathogenesis of ischemic lesions and the involvement of many double-way interconnected factors justifies the use of many means for prevention and treatment of ischemia in different organs.

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Making only 2% of the total body mass, the human brain utilizes about 20-25% of the oxygen used. Since the human brain contains high levels of lipids, rich in unsaturated links, ascorbate, which has antioxidant and pro-oxidant properties, low antioxidant system protection when compared to the other organs, it should be admitted the high probability of development of oxidant cerebral cell damage. When blood flow in the brain decreases down to 20 ml/100g of cerebral tissue in 1 min there is observed energy and "substrate" starvation, disorders in cellular ionic homeostasis, cell membrane depolarization, excessive release of excitatory amino acids (aspartate, glutamate), resulting in glutamate "excitotoxicity". As a result of the above-mentioned excessive accumulation of Ca^{2+} takes place in the cell cytoplasm (Gusev and Skvortsova, 2001).

A specific feature of taurine is its marked modulation of Ca^{2+}-dependent processes in the brain, heart and other organs. It stimulates Ca^{2+} transport and modulates Ca^{2+} channel activity by direct effect on a hydrophilic area near the channel, which also is under the influence of the majority of Ca^{2+} antagonists. Taurine decreases the cytoplasmic calcium level during the phase of repolarization and stabilization of cell membranes. Since the mechanisms of damaging effects of hypoxia and ischemia are in principle similar, the subdivision of drugs to antihypoxic and anti-ischemic is hardly justified. It should be recognized that the great majority of anti-ischemic remedies produce "multi-purpose" effect, influencing different stages and chains of pathologic processes in acute ischemia of organs.

Development of lactate-acidosis is one of the first tissue reactions upon the decrease in organ blood flow. The reduction of ATP content in the ischemic area results in compensatory activation of anaerobic glycolysis and increase of lactate and hydrogen ion (H^+) production, thus promoting development of metabolic acidosis. Intracellular acidosis leads to Ca^{2+} ion sequestration disorder in mitochondria and endoplasmic net due to the competition of H^+ and Ca^{2+} for binding places, accumulation of free intracellular Ca^{2+} ions and additional activation of the "trigger" pathogenic mechanisms: aggravation of the oxidant stress processes, excessive nitrogen oxide synthesis, and intracellular enzyme activation (Ooboshi *et al.,* 1992; Nakashima and Todd, 1996). Increased production of free radicals during acidosis is associated with the increased release of oxidative mechanism, which triggers liberation of iron from its binding to transferring-like proteins. This process intensifies reactions of free radical oxidation. It results in fast development of mitochondrial damage with the decrease of cytochrome oxidase activity and cytochrome content (Siesjö *et al.,* 1996).

At present, the inhibitors of free radicals include not only substances which interact with lipid radicals, i.e. real antioxidants (tocopherole, ubiquinones, 3- and 6-oxypyridines and their derivatives, vitamins K, PP, A, etc.), but also other substances, which are universal regulators of metabolic processes, inhibiting LP and closely connected processes of lipid hydrolysis, synthesis of prostaglandins, leukotriens, and enzyme systems which produce O_2 active forms. They include aliphatic and aromatic sulphur-containing compounds, ceruloplasmin, L-methionine, selenium-methionine, amino acids, nicotinic acid, etc. Thus in search of drugs to correct ischemic disorders, sulfur-containing taurine derivatives are rather important.

From this point of view, isopropylamide derivatives of taurine have a certain perspective. Altogether 26 new taurine derivatives were synthesized in the Department of Neuropharmacology named after S.V. Anichkov, the Research Institute for Experimental Medicine of the Russian Academy of Medical Sciences. Three substances of them were subjected to thorough studies: 2-(1-methyl-2-phenylethylamino)ethanesulfonic acid

isopropylamide hydrochloride (Taurhythman); 2-(benzylamino)ethanesulfonic acid isopropylamide hydrochloride (IEM-1702); and 2-(1-phenylethylamino)ethanesulfonic acid isopropylamide hydrochloride (Taurepar). These substances showed a wide range of biological activity, being hepatoprotective, antiaggregative, antiarrhythmic, anti-ischemic, antihypoxic, antioxidant, anti-inflammatory, reparative and hypolipidemic.

The above-mentioned effects were noted in pathologic models of hypoxia, arrhythmia, myocardial ischemia, cerebral and spinal cord ischemia, dyslipoproteinemia, aseptic layer-by-layer skin wounds, gastric and duodenal ulcer, colitis, and toxic hepatitis. In addition, the taurine derivatives possess low toxicity, normalize blood rheological properties, suppress platelet aggregation (Kudryashova *et al.,* 1987), produce membrane-stabilizing action (Sapronov *et al.,* 1999a), and immunotropic activity (Sapronov *et al.,* 2001).

In general, the data obtained show that the substances have multicomponent activity. Taurhythman was particularly cardioprotective and Taurepar exhibited neuroprotective, hepatoprotective, anti-inflammatory and reparative effects.

2. TAURHYTHMAN

The experiments in animals showed that this drug decreased arterial pressure, general peripheral resistance, cardiac output, and force and frequency of cardiac contractions. The substance could thus promote depressive shifts in hemodynamics (Sapronov *et al.,* 1998). In the models of circulatory and hypoxic hypoxia the drug produced antihypoxic (Sapronov *et al.,* 2004) and cardioprotective effects (Sapronov *et al.,* 1998). It diminished the necrotic area in experimental myocardial infarction (Sapronov *et al.,* 1999b), and possessed antiaggregate activity (was not less effective than acetylsalicylic acid) mainly directed on the irreversible stage of platelet aggregation (Kudryashova *et al.,* 1987). It produced an antiarrhythmic effect in the case of rhythm disturbances caused by the occlusion of the left coronary artery, introduction of aconitine and calcium chloride, being comparable the efficacy with procainamide hydrochloride and xylocain (Sapronov *et al.,* 1999a). The drug belongs to antiarrhythmic and membrane-stabilizing effectors, blocking sodium channels.

These properties of Taurhythman were the impetus to recommend it for clinical trials in the acute stage of myocardial infarction, ventricular arrhythmias: (except arrhythmias caused by digitalis intoxication), ventricular premature beats, paroxysmal ventricular tachycardia, and for prevention of ventricular fibrillation.

3. TAUREPAR

Antioxidant (AO) efficacy largely depends on the chemical structure, determining AO affinity to membranes necessary for interaction with oxidative substrates (fatty acid acyles of phospholipids). Besides the activity depends on the volume of AO molecule and the ratio of its hydrophobic and hydrophilic constituents, promoting embedding of the molecule in different membrane areas. The presence or absence of such functional groups as hydroxyl (OH^-) or groups influencing hydrogen atom mobility determines the activity of these substances (Sherstnev *et al.,* 1985). Some studies show that antioxidant activity of tocopherol analogues which have substitutes in the side chain decreases in the

sequence: C_1-C_3≥C_6>C_{11}-C_{16}. This is probably connected with the degree of their mobility in biomembranes (Kagan *et al.,* 1987).

Due to the amino and sulfonic groups in the taurine molecule, this amino acid is a highly active natural compound. Its effects on the functional body systems may be in general considered as adaptogenic. Taurine suppresses LP, protects hepatocytes from the toxic influence of tetrachlormethane, (Huxtable, 1992), and protects the retina from oxidant effects (Lima *et al.,* 2001). However, many authors consider taurine actions result from its precursors hypotaurine and cysteamine. Taurine introduced into the organism has only membrane-stabilizing and osmoregulatory activity.

Our studies showed that unlike of taurine, the antihypoxic and antioxidant properties of taurinamide derivatives were connected with the presence 1-(2-phenylalkyl) group attached to the amino group and N-isopropylsulfonamide radical. Such substitution provided its penetration through the blood-brain barrier, enhanced CNS stability against external damaging effectors and improvements of mnestic processes.

A comparative study of Taurepar with piracetam and xantinol nicotinate in brain ischemia showed that it was similar to xantinol nicotinate in its action. Unlike piracetam the compound increased the survival rate of animals during 14 days. During the whole study the drug exhibited neuroprotective activity, promoted normalization of disturbed energy metabolism, restored the activity of the enzymes of antioxidant system (superoxide dismutase and catalase), decreased the intensity LP after brain ischemia within 3 days after arterial occlusion. This may prove the antioxidant activity of Taurepar and its rapid involvement in the regulation of hydroxyl radical formation at the stage of LP initiation.

Taurine and GABA modulators have an important role in regulation of synaptic transmission in the stress-limiting systems providing adaptation. Taurine has been shown to be involved into the processes of learning and memory. It plays an important role in long-term potentiation. This amino acid improves memory in mice treated with a variety of amnestic agents, such as pentobarbital, cycloheximide, sodium nitrite and alcohol. Taurine also improves memory in rats exposed to ozone (Rivas-Arancibia *et al.,* 2000). However, it does not improve memory in healthy untreated mice (Vohra and Hui, 2000). Taurine increases the levels of acetylcholine in the brain of animals, and decreased levels of taurine have been found in patients with Alzheimer's disease (Birdsall, 1998). It is to be seen whether taurine improves memory in humans, but it is likely to have an effect at least in individuals with impaired memory.

In the rat experiments, intraperitoneal Taurepar (25 mg/kg) produced positive effects on mnestic functions and exhibited antiamnestic activity after electroconvulsive shock or after intraperitoneal introduction of pentetrazol and scopolamine. The drug prevented disorders in mental activity, and increased adaptive abilities in the CNS and elsewhere in the body in different pathologic states, e.g., amnesia, brain ischemia and hypoxia (Sapronov *et al.,* 2004). The protective activities of Taurepar and IEM-1702 were studied in ischemic and stress lesions of the cardiovascular and gastroduodenal systems (Sapronov *et al.,* 2000; Khnychenko *et al.,* 2002; Zavodskaya *et al.,* 2002). The hypolipidemic effect of these drugs was established in experimental dyslipoproteinemia, which was manifested by the decreased level of cholesterol and the considerable decrease in the triglyceride level in blood serum (Sapronov *et al.,* 2004).

Taurepar shows desintoxication and reparative effects on rat liver damage induced by tetrachlormethane. It normalized the activity of transaminase enzymes, glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT); – markers of hepatocyte damage, and diminished hyperbilirubinemia. Taurepar and IEM-1702

inhibit LP and increase the antioxidant protection in the liver (Khnychenko *et al.,* 2001). When these substances studied were given intraperitoneally to intact animals at a dose of 25 mg/kg during 21 days, no unfavorable effects were discernible in the parameters of protein, carbohydrate and lipid metabolism. When the influence of taurine derivatives on lipid metabolism was studied in rats during enhanced lipolysis stimulated by epinephrine, it was found that preliminary introduction of the compounds during the 10-day period produced antilipolytic actions manifested in the decrease of non-estherified fatty acids (NEFA) in blood serum.

In conclusion it is necessary to emphasize that taurinamide derivatives showed extreme polymorphism in their pharmacological properties. Both peripheral and central activity is quite characteristic of them. The results show that compounds belonging to the class of taurine derivatives are promising when searching of novel highly effective drugs.

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POTENTIAL ANTIATHEROSCLEROTIC DRUGS: NOVEL N-SUBSTITUTED TAURINAMIDE DERIVATIVES

Nikolay S. Sapronov, Ludmila K. Khnychenko, Irina V. Okunevich, and Ludmila K. Gavrovskaya*

1. INTRODUCTION

Hyperlipoproteinemia or dyslipoproteinemia (DLP) is one of the basic factors resulting in symptomatic atherosclerosis (A), which is chronic multifactorial disease (ACC/AHA, 2001; Adult Treatment Panel III, 2001). Dietary measures and physical activity are commonly combined with drug treatment for high-risk individuals. DLP and such cardiovascular diseases as atherosclerosis-related coronary heart disease (CHD), arterial hypertension and stroke are the main causes of mortality in many countries (Kwiterovich, 1998; Klimov and Nikulcheva, 1999). Prevention of cardiovascular diseases related to A is based on the screening for risk factors and their control, recommended dietary regimens, changes in lifestyle, and pharmacotherapy (Kwiterovich, 1998; Smidle, 2001; Warlow and Chalmers, 2001). The antiatherogenic action of high-density lipoproteins (HDL) implies that HDL serves as scavengers for exchanged tissue cholesterol (Ch) (Klimov and Nikulcheva, 1999; Smidle, 2001). High HDL-Ch has been associated with the reduced risk of CHD development in patients. Pharmacological approaches to raise low HDL-Ch levels are very important (ACC/AHA, 2001; Adult Treatment Panel III, 2001). Intensive treatment of enhanced levels of atherogenic lipoproteins, low- and very low-density lipoproteins (LDL/VLDL), may prevent future chronic clinical complications (Cameron *et al.,* 2001; Smidle, 2001). The physiologically active substance taurine, a sulfur-containing beta-amino acid, is not only a natural membranoprotective agent, but has the property to antagonize the oxidative effects of homocysteine, the marker of cardiovascular diseases (Tanne *et al.,* 2003; Chang, *et al.,* 2004). Recently, it was shown that taurine prevents the progression of A caused by different pathogenetic mechanisms. Treatment with taurine inhibited lipid peroxidation,

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lowered atherogenic serum LDL/VLDL cholesterol and elevated antiatherogenic HDL (Obrosova *et al.,* 2001; Ito and Azuma, 2004).

In this work the hypolipidemic activity of new original taurine derivatives Taurepar and Taurhythman was investigated. They were synthesized in the Department of Neuropharmacology. Previous investigations showed that all compounds tested were able to limit hypercholesterolemia induced with triton WR-1339 in rats. Taurepar and Taurhythman also effectively decreased triglycerides in blood serum.

2. MATERIALS AND METHODS

At the beginning experiments carried out to study the possible metabolic effectiveness of taurinamide derivatives Taurepar (Trp) and Taurhythman (Trm). We used the model of starvation. Eighty male Wistar rats were fed with a diet reduced down to 25% from the standard one. When the body weight was decreased to about 35% (nutritional dropsy) all animals were divided into control and experimental groups. The control group was fed with the standard diet enriched with soybean protein. The experimental group received the same diet, but additionally the animals were treated with taurine derivatives at the intraperitoneal dose of 25 mg/kg of body weight. Glucose, total Ch (TCh), triglycerides (TG), and non-esterified fatty acids (NEFA) were measured in blood serum.

Special experiments in the alimentary cholesterol-rich diet model were carried out to detect the hypolipidemic action of new taurine derivatives. Two hundred albino male rats (adult, 230 g) and 60 male guinea pigs (adult, 500 g) were used in the experiments. These animals were kept under constant conditions: humidity, and temperature 20 ± 2.0°C, and fed normal standard laboratory diet. All our investigation conforms to the Guide for the Care and Use of Laboratory animals published by US National Institute of Health. Before the experiments all animals (rats and guinea pigs) were distributed randomly according to their weight in five groups of 8-10. The first group was fed the control diet and the second the high-cholesterol diet (ChRD) containing 7.5% and 50% saturated natural lipids. The **o**ther groups 4 and 5 were fed the same ChRD and additionally orally Trp and Trm 50 mg/kg. The well-known hypolipidemic drug gemfibrosil (Gfb, Gevilon, "Lechiva", Czech Repablic) was used for comparisons. Gfb 50 mg/kg was given to the control ChRD group 3. The model of experimental DLP in guinea pigs lasted 16 days and in rats 21 days. After starvation during 18 hours the animals were killed by decapitation in the morning between 8 and 9 hours to avoid circadian variations in sensitive lipids.

The samples of blood were collected from the neck and serum was obtained by centrifugation at 3000 g for 10 minutes. The aortas and livers were removed, weighed, and homogenized in ice-cold 0.9% NaCl. Lipids of the aorta (TCh) and liver (TCh and TG) were examined after extraction (Okunevich *et al.,* 1999). The serum concentrations of TCh, TG, and HDL-Ch were analyzed spectrophotometrically using special chemicals (Boehringer Manheim, Germany). TCh was determined by the colorimetric test cholesterol CHOD-PAP and TG assayed by means of a Peridochrom Triglyceride GRO-PAP test. Ch-HDL we tested after sedimentation of VLDL and VDL using a precipitation method (solutions of $MnCl_2$ and geparine). The concentrations of glucose and NEFA were measured in blood serum by the colorimetric method GOD-PAP tests. All chemicals were obtained from commercial sources and were of analytical grade. The results are expressed as means with S.E.M. of three experiments performed in duplicate.

The data obtained were analyzed with Student's t-test using statistical program SPSS 11.5.

3. RESULTS

Metabolic disorders after starvation and their pharmacotherapy with taurine derivatives Trp and Trm are shown in the Table 1. This table shows the variations of concentrations of glucose, NEFA, TCh, and TG with respect to control values before starvation.

Table 1. Nutritional dropsy (ND) and treatment of taurine derivatives in rats

Group of animals	Serum lipids (mmol/l)			Serum glucose (mmol/l)
	Total Cholesterol	Triglycerides	NEFA	
1. Intact rats	2.10 ± 0.08	1.07 ± 0.05	237.3 ± 46.6	5.7 ± 0.4
2. Nutritional dropsy	1.20 ± 0.17*	0.76 ± 0.05*	623.0 ± 120.0*	2.9 ± 0.6*
3. ND+full ration	2.30 ± 0.14[a]	0.93 ± 0.03 [a]	480.0 ± 29.7*[a]	5.3 ± 0.5[a]
4. ND+full ration+Trp	2.20 ± 0.13[a]	0.93 ± 0.06 [a]	305.0 ± 68.4[ab]	5.5 ± 0.4[a]
5. ND+full ration+Trm	2.40 ± 0.10[a]	0.92 ± 0.04 [a]	270.0 ± 21.8[ab]	5.7 ± 0.5[a]

The values are means with S.E.M.* Data are positively different in comparison to the intact group ($p<0.05$), [a] to the nutritional dropsy group 2 ($p<0.05$), and [b] to group 3 ($p<0.05$).

The results obtained in the model of starvation show that nutritional dropsy led to a significant decrease in glucose, TCh, and TG, as well as to an increase in NEFA in blood serum.

In the rats with nutritional dropsy (group 3) fed with standard diet enriched with soybean protein the body weight returned to normal values and the levels of glucose, TCh, and TG were restored. However, the concentration of NEFA remained increased (Fig. 1, group 3). In the experimental groups 4 and 5, which received additionally Trp or Trm, the level of NEFA decreased to normal. It is necessary to mention that taurine derivatives did not change the biochemical parameters in blood of normal non-starved rats.

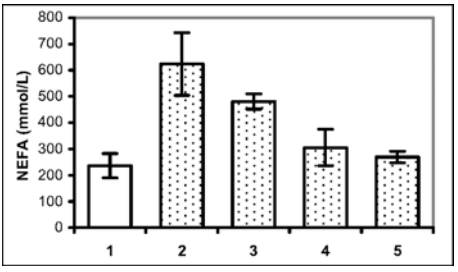

Figure 1. Serum blood concentration of NEFA in rats after starvation. 1 – intact animals; 2 – nutritional dropsy (ND); 3 – ND + normal standard diet; 4 – ND+normal standard diet+Taurepar; 5 – ND+normal standard diet+ Taurhythman.

In accordance with the data obtained, the special experiments using the alimentary cholesterol-rich diet model were carried out to detect the hypolipidemic action of new taurine derivatives.

Table 2 shows that the guinea pigs fed with ChRD during 16 days had significant differences from the control group (normal diet). The serum levels were increased: TCh was 6.6-fold and TG 7-fold higher than in the normal diet (group 1). Taurepar and gemfibrosil treatment produced hypolipidemic effects, both reducing serum TCh (27% and 42%, respectively) and serum TG (37% and 45%, respectively). These values in the drug-treated groups were statistically significant at the level of $p<0.05$. Taurhythman decreased only TCh (34% *vs.* the ChRD group) and did not produce TG reduction under the same conditions (Table 2).

Table 2. Serum blood lipids of guinea pigs receiving cholesterol-rich diet (ChRD) during 16 days

		Serum lipids (mmol/l)	
		Total cholesterol (TCh)	Triglycerides (TG)
1.	Normal diet	1.23 ± 0.10	0.44 ± 0.05
2.	Group of animals	$8.15 \pm 0.62^{*}$	$3.10 \pm 0.18^{*}$
3.	ChRD + Gfb	$4.73 \pm 0.35^{*a}$	$1.72 \pm 0.3^{*a}$
4.	ChRD + Trp	$6.00 \pm 0.37^{*a}$	$1.96 \pm 0.1^{*a}$
5.	ChRD + Trm	$5.43 \pm 0.34^{*a}$	$2.85 \pm 0.16^{*}$

The values are means with S.E.M. * Data positively different from the normal diet ($p<0.05$) and ^{a} from group 2 ($p<0.05$)

Table 3 concerns results obtained with the rats receiving ChRD during 21 days. The blood serum lipid levels were raised to a rather significant degree: TCh and TG were 3-fold higher than in the normal diet (group 1). It is important to mention that antiatherogenic HDL-Ch was 1.7-fold lower than normal. The absolute concentrations of HDL-Ch were in 41% (Gfb) and 19% (Trp) higher than in the CHRD group. Treatment with Trm was not effective to raise HDL-Ch. In spite of these findings, the evaluation of the atherogenity index which was calculated using TCh and HDL-Ch (TCh–HDL-Ch)/(HDL-Ch) showed a positive effect. All the drugs significantly decreased this parameter: gemfibrosil 3.35, Taurepar 3.72 and Taurhythman 4.92 when compared to 9.43 in the cholesterol-rich diet group (Table 3).

Fig. 2 summarizes the results of aorta lipidosis in the guinea pigs and rats fed ChRD. Absorption of dietary Ch was significantly reduced in each case after Gfb, Trp, or Trm treatments for 16 days (guinea pigs) and 21 days (rats). Feeding of ChRD to guinea pigs caused TCh to be 6-fold higher than upon normal diet (group 1). The TCh content in the aortas was lower; 27% (Gfb), 20% (Trp) and 15% (Trm), when compared to the ChRD group (Fig. 2). Similar results were obtained with rats but the reduction in TCh was more pronounced: 56% (Gfb), 34% (Trp), and 47% (Trm).

Table 3. Serum lipids in blood of rats receiving cholesterol-rich diet (ChRD) during 21 days

Group of animals		Serum lipids (mmol/l)			Index of atherogenity
		TCh	HDL-Ch	TG	
1.	Normal diet	1.29 ± 0.06	0.64 ± 0.04	0.53 ± 0.06	1.02
2.	ChRD	3.86 ± 0.39*	0.37 ± 0.01*	1.67 ± 0.06*	9.43*
3.	ChRD+ Gfb	2.70 ± 0.16*a	0.62 ± 0.07*a	1.23 ± 0.09*a	3.35*a
4.	ChRD+Trp	2.36 ± 0.16*a	0.50 ± 0.05*a	1.04 ± 0.11*a	3.72*a
5.	ChRD+ Trm	2.31 ± 0.22*a	0.39 ± 0.07*	1.04 ± 0.13*a	4.92*a

The values are means with S.E.M. * Data positively different from the normal diet ($p<0.05$) and [a] from group 2 ($p<0.05$)

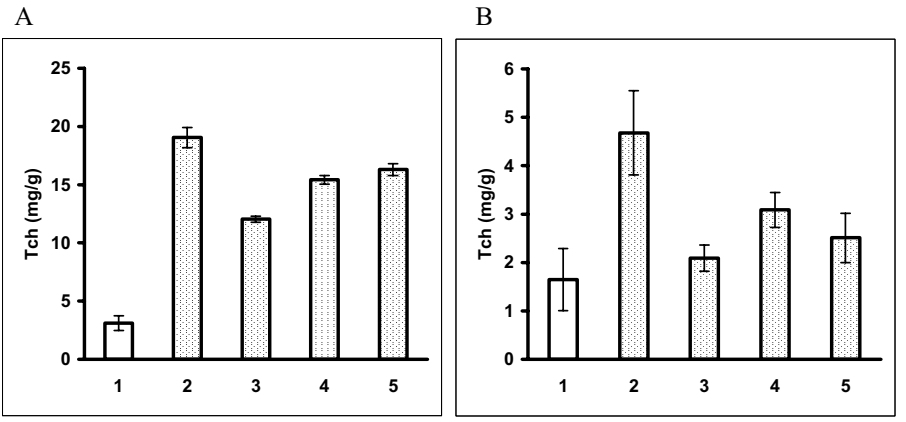

Figure 2. Total cholesterol content in the aorta of guinea pigs fed ChRD during 16 days (A) and rats fed ChRD during 21 days (B). 1 – control diet, 2 – ChRD (cholesterol-rich diet), 3 – ChRD+Gfb, 4 – ChRD +Trp, 5 – ChRD+Trm.

Table 4 shows the results of liver lipidosis in two models of alimentary DLP (ChRD in guinea pigs and rats). It is evident from the data that the lipid content in the liver of guinea pigs is 7 times higher in the group with ChRD than in the control group (normal diet, group 1). The content of TCh in the liver in the Taurepar- and Taurhythman- treated groups was reduced to 37% and 34%, respectively. However, Taurhythman treatment did not decrease the liver content of TG (Table 4, group 5). There was a 40% reduction in the liver TG content in the Taurepar- and gemfibrosil-treated animals (Table 4, groups 3 and 4). The analysis of data obtained in the rat model of DLP showed that liver lipidosis in the ChRD group was less expressive than in the guinea pig model. The lipid content in the liver was only 3.4 (TCh) and 4.8 (TG) times higher than in the ChRD group. As expected, all three drug-treated groups showed reduction of liver lipidosis: TCh was 76%, 77%, and 79% lower (Gfb, Trp, and Trm, respectively) than in the ChRD group. Similar results were also observed in the liver TG content: 67%, 56%, and 57%, respectively. The control liver lipid content was higher in the ChRD group of guinea pigs than in the ChRD group of rats.

Table 4. Lipid content of liver in guinea pigs and rats fed Cholesterol-rich diet (ChRD) during 16 and 21 days respectively (means with S.E.M.)

Group of animals		Model of DLP			
		Guinea pigs		Rats	
		Liver lipid content (mg/g)		Liver lipid content (mg/g)	
		TCh	TG	TCh	TG
1.	Normal diet	4.2 ± 0.30	5.3 ± 0.5	3.4 ± 0.24	4.1 ± 0.35
2.	ChRD	30.2 ± 2.7*	35.1 ± 3.6*	11.6 ± 0.6*	19.7 ± 1.7*
3.	ChRD+Gfb	17.8 ± 2.2*a	21.3 ± 4.5*a	2.79 ± 0.39*a	7.56 ± 0.64*a
4.	ChRD+Trp	19.2 ± 3.4*a	23.1 ± 2.6*a	2.71 ± 0.70*a	8.69 ± 0.28*a
5.	ChRD+Trm	20.1 ± 3.1*a	26.5 ± 3.7*	2.44 ± 0.36*a	8.64 ± 0.38*a

*The data are positively different from the normal diet ($p<0.05$) and [a] from the group 2 ($p<0.05$)

4. DISCUSSION

In spite of the wide spectrum of the protective effects of taurine, its pharmacological application is limited by a low comparatively activity and a weak ability to penetrate across the blood-brain barrier. That is why the creation of new taurine containing compounds and the investigation of dependence between chemical structure and their pharmacological activity are very actual tasks. Previous experiments on the rat's model of starvation showed the ability of taurine amide derivatives Taurepar and Taurhythman to decrease the serum concentration of NEFA. We suppose that these new substances promote the reduction of intensity of hyperlipidemic processes. It is known that during starvation incomplete oxidation of fatty acids leads to acidosis with following destruction of mitochondrial membrane. The property of taurine derivatives to decrease the concentration of non-estherified fatty acids points to their ability for restoration of tricarboxylic acid cycle and to the prevention of accumulation of suboxized molecules of NEFA and development of acidosis (Khnychenko, 2001). The data obtained in the first special experiments to study lipid metabolism showed the positive hypolipidemic effects of Taurepar and Taurhythman in screening models of DLP induced by the detergent Triton WR-1339. These results initiated the following study on the effectiveness of new original substances in the alimentary models with cholesterol-rich diets. We used the lipid-lowering agent gemfibrosil (Gfb) for comparison to our new substances. Gfb is a medicine of the fibrate group. Fibrates have been shown to reduce the TG-rich lipoprotein levels (VLDL and LDL) and to increase the HDL-cholesterol (HDL-Ch) levels (Kwiterovich, 1998; Klimov and Nikulcheva, 1999;). Studies on experimental animals have shown that fibrates reduce the fatty acid contents in the liver and cause a significant reduction in the plasma cholesterol and triglycerides levels in guinea pigs (West and Fernandez, 2004). In the present study we observed the similar action of taurine derivatives. This research demonstrated that Taurepar is more effective than Taurhythman in the reduction of TG liver lipidosis in guinea pigs. It is possible because of differences in the lipid profiles in guinea pigs and rats. Like humans, the guinea pig is one of the few species that carry the majority of cholesterol in LDL (West and Fernandez, 2004). Our results in the other series of experiments with rats suggested positive hypolipidemic effects of Taurhythman and Taurepar in guinea pigs. It is very important fact because the lipid profile of blood serum in rats differs from that in guinea pigs. These animals have a high level of antiatherogenic HDL-Ch. Rats have been shown to be not useful for chronic testing. Scientists have had many difficulties to produce experimental

hyperlipidemia and atherosclerosis. The second finding of our study is the ability of taurine derivatives to decrease significantly the cholesterol index of atherogenity. The index of atherogenity has the prospective value in estimation of progression of atherosclerosis in the investigations, choosing new hypolipidemic drugs (Klimov and Nikulcheva, 1999). The most interesting finding is that Taurepar and Taurhythman had a significant effect to reduce the TCh content in the aortas of rats and guinea pigs. Probably this property was connected with the action of taurine and its derivatives to decrease the intensity of oxidative stress (Obrosova *et al.,* 2001). We can suppose and conclude that the new taurinamide derivatives Taurepar and Taurhythman are potential active hypolipidemic and antiatherosclerotic drugs.

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ANTIHYPOXIC PROPERTIES OF TAURINAMIDE DERIVATIVES: THE EXPERIMENTAL STUDY

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1. INTRODUCTION

Disorders of the brain and other system activity in hypoxia are found not only in different human diseases, professional occupations, but they are also due to the influence of the disturbed ecology. Search for antihypoxants, compounds correcting deficient oxygen state and increasing organ and tissue resistance to hypoxia, remains quite urgent. Taurine regulates nervous and biochemical processes in CNS and other systems, stabilizes membrane permeability and ion transport, increasing organ and tissue resistance to anoxia/hypoxia, prevents intracellular membrane damage (Franconi *et al*., 1985; Huxtable, 1992; Mankovskaya *et al*., 2000).

The aim of this work was to study antihypoxic properties of amide derivatives of taurine: Taurhythman, Taurepar and IEM-1702. These substances exhibit antioxidant (Sapronov *et al.,* 2004) and antiaggregation properties (Kudryashova *et al*., 1987), and possess hepatoprotective (Khnychenko *et al*., 2002) and antiulcerative effects (Sapronov *et al*., 2000).

2. METHODS

2.1. Hypoxic Models

Normobaric hypoxic hypoxia with hypercapnia was reproduced in mice weighing 19 g, which were placed individually in hermetically closed jars with the volume of 200 cm^3. Hemic hypoxia in mice (18-22 g) was induced by the subcutaneous introduction of 40

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mg/kg sodium fluoride. Hypobaric hypoxic hypoxia was performed in Wistar male rats weighing 250-300 g. The animal behavior was examined when "rising" to the height of 11000 m with the speed of 25 m/s in the pressure chamber. During the experiment the control animals were kept in a cage at the normal pO_2 level. The number of the dead animals and the time of their death were monitored.

In chronic hypoxic hypoxia of male Wistar rats weighing 250-300 g, the activity of membrane-bound acetylcholinestarase enzyme (AchE) was studied in order to obtain information of the state of cholinergic neurons. The rats were exposed to hypoxia in a special chamber with the volume of 100 liters, which contained systems of thermoregulation, ventilation, adsorption of the expired CO_2 and gas analyzer. During the experiment, the oxygen content in the chamber was reduced from 20 to 7.8% (this level corresponds to the oxygen content at the height of 8000 m) within the 30-min period and was maintained at this level for another 2.5 hours. The carbon dioxide concentration in the chamber was not higher than 0.1% at 22°C. In all models, Taurhythman, Taurepar and IEM-1702 were injected intraperitoneally in the optimal dose of 25 mg/kg 20 min before the beginning of hypoxia (Ivanova and Bobkov, 1984). Standard antihypoxant gutimin (Vinogradov *et al*., 1981) in dose 25 mg/kg and piracetam 100 mg/kg were used as reference compounds. The control animals were given only saline. After hypoxia, cerebral synaptosomes and membrane fractions of cardiac muscle and stomach were prepared and their AchE analyzed following the method of Ellman *et al.* (1961), and the content of the protein was determined according to the Lowry method.

The synaptosomes were isolated from the cerebral hemispheres. All the procedures were performed at 4°C. Tissue homogenates in 0.32 M sucrose solution in 0.01 M tris-HCl (pH 7.4) were centrifuged for 10 min at 1000 g. The sediment was suspended in the initial solution of sucrose and re-centrifuged as above. The united supernatant was centrifuged for 20 min at 20000 g, the deposit suspended in the initial sucrose solution and re-centrifuged in the gradient of the sucrose concentration at 100000 g for the 1-hour period. The synaptosome fraction, formed at the border of 0.8 M and 1.2 M saccharose solutions, was sucked off and centrifuged for 20 min at 20000 g. The synaptosome sediment was twice washed in 0.01 M tris-HCl (pH 7.4) and centrifuged. The washed sediment of synaptosomes was suspended in 2 ml of 0.01 M tris-HCl (pH 7.4).

The membrane fraction of the cardiac muscle was isolated following the method of Avdonin *et al*. (1980). Cardiac muscle was weighed, placed in 20 mM tris-HCl (pH 7.4), fragmented by scissors, and homogenized. The homogenate was filtered through two-later gauze and repeatedly centrifuged at 1600 g for 10 min. After each centrifugation the sediment was homogenized in 30 ml of 20 mM tris-HCl (pH 7.4). The washings continued until complete clearing of the supernatant fluid. The sediment membrane preparation thus obtained was suspended in 10 ml of the initial buffer.

The membrane fraction of the stomach was obtained following the above scheme for the cardiac muscle with some modifications. The supernatants obtained after the first two centrifugations of the gastric homogenate in 20 mM tris-HCl, were united and centrifuged for 20 min at 500 g and 13°C. The resultant sediment was suspended in 5 ml of the initial buffer. The results were analyzed using t-test and the method of paired comparisons.

2.2. Vibration Influence on Cochlea

Circulatory hypoxia in guinea pigs weighing 250-300 g was produced by general vertical vibration. Maximal meanings of the vibration were the mean geometrical frequencies of octave strips from 31.5 Hz to 63 Hz and equaled to 72 dB and 82 dB, respectively. The work of the vibrostend was accompanied by a permanent noise with the level of sound pressure 88 dB. The duration of vibration was 3 hours every day during 4 weeks. After the decapitation of animals the cochleae were removed for the following morphological investigations and the bullas dipped into fixing solution (Vinnikova and Titova, 1959). The ultrathin slices were analyzed with electron microscope TESLA-BS-540 and the reticular plate of Corti's organ was investigated in scanning electron microscope JSM-T-300. The basal, middle and apical parts of the guinea pig cochleae were studied in three groups of animals: (1) control (intact) animals; (2) guinea pigs after 4-week vibration; and (3) animals receiving Taurepar (10 mg/kg, i.m.) during 7 days before vibration.

2. 3. Study of Progeny Quality

In the experimental groups, Taurhythman was introduced orally in doses of 10, 25 and 50 mg/kg during the whole period of pregnancy. The control groups consisted of intact pregnant animals (passive control) and rats receiving saline in a volume of 0.2 ml. Psychophysiological characteristics of the progeny in the postnatal period were evaluated by physical state, rate of maturation of sensory-motor reflexes during suckling, emotional motor behavior, training, and memory according to international standards GLP.

3. RESULTS

3.1. Effects of Taurinamide Derivatives on the Types of Acute Hypoxia

In normobaric hypoxic hypoxia with hypercapnia, Taurepar, Taurhythman and IEM-1702 produced antihypoxic effect (Table 1). The same effect was noted for two other types of hypoxia. In hemic hypoxia, Taurepar and Taurhytman were most effective, while in hypobaric hypoxic hypoxia IEM-1702 and gutimin revealed the highest activity. With regard to their efficiency Taurepar and Taurythman are not less effective than gutimin in different types of hypoxia.

3.2. Effects of Taurineamide Derivatives on the Activity of AChE in Rats with Chronic Hypoxia

Chronic hypoxic hypoxia resulted in a decrease in the acetylcholinestarase enzyme activity in the brain synaptosomes and membrane preparations of rat hearts. Taurhythman prevented this activity decrease in heart membrane preparations (Table 2).

The data obtained show that three-hour-long hypoxia reduced the brain AChE activity (Table 2). The AChE activity decrease coincided with the lowering of motor activity in rats. Introduction of preparations (gutimin, Taurhythman, IEM-1702, and Taurepar) not only prevented the decrease in the AChE activity, but increased the activity of the enzyme when compared with the control. Previously, it was reported that the most

Table 1. Effects of taurinamide derivatives on acute hypoxia

Groups, n=10	Normobaric hypoxic hypoxia in mice	Hemic hypoxia in mice		Hypobaric hypoxic hypoxia in rats	
	Life time, min	Life time, min	Survival rate, %	Life time, min	Survival rate, %
Taurhythman 25 mg/kg	17.3± 0.5*	212 ± 14**	50 ± 17*	9.7 ± 2.3*	0 ± 8
IEM-1702 25 mg/kg	14.5± 0.9*	161 ± 16**	30 ± 15	15.2 ± 5.2**	25 ± 13
Taurepar 25 mg/kg	14.7 ± 0.6*	174 ± 15**	40 ± 16*	9.3 ± 2.0*	0 ± 8
Gutimin 25 mg/kg	16.5 ± 0.7*	148 ± 19**	30 ± 15	19.7± 5.5**	33 ± 15
Control (saline)	12.2 ± 0.6	54 ± 4	0 ± 10	3.3 ± 0.7	0 ± 8

*p<0.05; ** p<0.01 in relation to control.

important biochemical changes in the subcellular fraction are connected with disorders of cerebral supply with oxygen (Wright *et al*., 1986). The brain is very sensitive to the decreased oxygen content. The hippocampus, neocortex, and striatum, which participate in the regulation and realization of motor behavior of mammals, are primarily affected (Smith *et al*., 1984). A 2-fold decrease in the oxygen concentration resulted in significant memory worsening, reduction in training and working capacity, retinal lesion, motor activity disorder, and ototoxic action (Luft, 1965; Lukianova, 1990).

Hypoxic hypoxia decreased the heart AChE activity but had no effect on the activity of this enzyme in gastric membranes. Taurhythman prevented the AChE activity decrease in membranous heart preparations; the effect not found with all other preparations (Table 2). Besides introduction of gutimin, Taurhythman and IEM-1702 resulted in a significant increase in the AChE activity in gastric membranes when compared to the control.

Table 2. Effects of taurinamide derivatives on the activity of acetylcholinesterase in rats with chronic hypoxia

Groups, n=10	AChE in brain synaptosomes μmol/mg protein/min	AChE in cardiac membranes μmol/mg protein/ min	AChE in gastric membranes μmol/mg protein/min
Control	0.1305 ± 0.0045	0.0893 ± 0.0038	0.0413 ± 0.0019
Hypoxia	0.1028 ± 0.0032*	0.0612 ± 0.0039*	0.0409 ± 0.0022
Hypoxia + Gutimine	0.1477 ± 0.0014*	0.0629 ± 0.0009*	0.0558 ± 0.0030*
Hypoxia + Taurhythman	0.1515 ± 0.0031	0.0826 ± 0.0033	0.0645 ± 0.0036
Hypoxia + IEM-1702	0.1536 ± 0.0029	0.0557 ± 0.0022*	0.0570 ± 0.0020
Hypoxia + Taurepar	0.1383 ± 0.0019*	0.0699 ± 0.0027*	0.0370 ± 0.0018

* p<0.01 in relation to control.

3.3. Effect of Taurepar on the Spiral Organ in Guinea Pigs with Circulatory Hypoxia

In scanning electron microscope, considerable pathologic alterations were observed in the reticular lamina of the apical part of cochlea in the guinea pigs exposed to vibration. In the other part of cochlea the changes were less expressed. There was a "smoth out" of the apical side of the supporting cells because of the fusion of the microvillia. On the apical side of the sensory cells a marked number of hernial bulges was noted. The outer hair cells did not have so much "hernias" as inner cells. These alterations can be explained by disturbances of metabolic processes in the inner ear; a metabolic stress development after vibration exposure (Bhattacharyya and Dayali, 1991; Lantsov *et al*., 1996). For both types of receptor cells there was a characteristic destroy of connections between separate stereocilia in each bundle and disturbances in the order of bundles (Fig. 1A). In the reticular lamina of Corti's organ of guinea pigs preliminary treated with Taurepar (Fig. 1B) some interruption of connections between stereocilias and fusion of microvilli were also observed. However these changes on the side of the hair cells were considerably less expressed than in group 2 (vibration without drug). On the surface of supporting cells there was no fusion of microvilli or decrease in their amounts. Some alterations in the ultrastructure of sensitive cells (such as the extension of cisterns of subsuperficial net or vacuolization of a small part of mitochondria) can represent reversible changes. Thus, Taurepar possesses the property to stimulate energetic processes in tissues of the organism; in particular to activate metabolic processes in the cochlea defending the structures of the internal ear against the damaging influences of vibration.

3.4. Effect of Taurhythman on Progeny Quality

Taurhythman in a dose of 50 mg/kg produced a positive effect on the development of conditional reflex in the Y-shaped labyrinth with food reinforcement in 3-month-old progeny. The animals reached the training criterion earlier than the controls. The percent of correct responses on days 2, 3 and 4 of training in the experimental group was significantly higher than in the control group. Positive transplacental action on training of rats of the new generation is probably connected to antihypoxic Taurhythman activity.

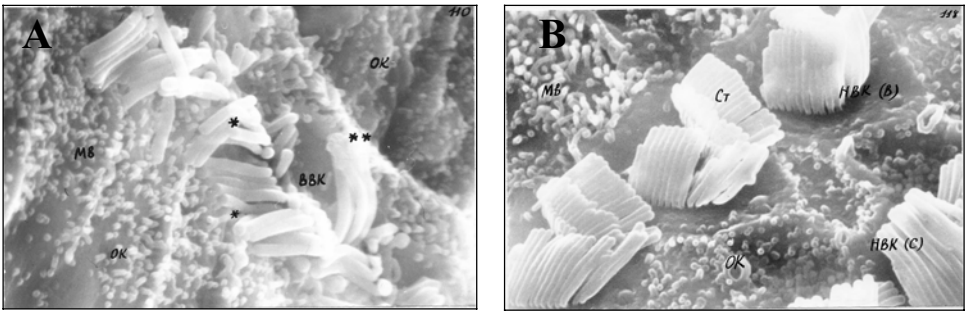

Figure 1. Reticular lamina of Corti's organ (apical part of the cochlea) of the guinea pig. (A) One month after the vibration stopped; and (B) preliminary treatment with Taurepar (10 mg/kg daily for 7 days). Ст stereocilia; Mb microvilli; OK supporting cells. Blow-up 8200.

Taurhythman treatment during the whole period of pregnancy apparently promoted the improvement of placental circulation, prevented development of hypoxic states in both antenatal and postnatal periods of rat development and produced positive influence on training and memory of the new generation.

Thus, in the different models Taurhythman, IEM-1702 and Taurepar revealed antihypoxic properties and produced protective effects on organs and systems of experimental animals under extreme conditions.

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THE INFLUENCE OF A TAURINAMIDE DERIVATIVE ON SKIN WOUND HEALING IN RATS: THE EXPERIMENTAL STUDY

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1. INTRODUCTION

It is known that taurine (2-aminoethanesulfonic acid), an amino acid containing sulfur and playing various important physiological roles in the organism, is found in almost all tissues in mammals. Taurine influences the membrane structure and the functions of tissues and cells (Huxtable, 1992), and exhibits antioxidant and antiaggregative properties. Taurine is also known to have effects on cell proliferation, inflammation and collagenogenesis (Degim *et al*., 2002).

The aim of the present study was to examine the wound-healing effect of a taurinamide derivative 2-(1-phenylethylamino)ethanesulfonic acid isopropylamide hydrochloride (Taurepar) in rats. This substance exhibits antihypoxic, antioxidant (Khnychenko *et al*., 2002; Sapronov *et al*., 2004), and antiaggregative properties (Kudryashova *et al*. 1987), as well as possesses hepatoprotective (Khnychenko *et al*., 2002) and antiulcerative effects (Sapronov *et al*., 2000).

2. METHODS

A total of 125 adult male non-linear white rats weighing 200-250 g were used. The experiments were carried out in wintertime. Under ether anesthesia the back of experimental animals was sheared and a round shred of skin 3.0 cm^2 was cut off according to the standard technique (Zapadnuk *et al*., 1983).

After operation each animal was housed in an individual cage for 35 days under the standard conditions. All rats with inflicted wounds were randomly divided into 4 groups,

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(n=25), «control group» (untreated rats), «placebo» (lanoline–vaseline 1:1), «Solcoseryl 20% gel» and «Taurepar 5% ointment». Treatment of wounds started in a day after wound infliction and continued daily up to complete healing.

Efficiency of the drug was estimated by the general state of animals, change in body weight, planimetric data, and dynamics of wounds healing and their morphological picture. The animals were weighted before feeding once a week. The wound area was measured during the experiment on days 5, 10, 15, 20, and 25. The imprint of wound was scanned and its area calculated with a special computer program elaborated in our Department of Neuropharmacology. The animals were sacrificed when the experimental protocol was completed.

For histological study wound biomaterial was taken on the 5th, 10th, 15th, and 20th days of the experiment. These time periods correspond approximately to three phases of wound healing (the 5th day corresponds to the phase of acute traumatic inflammation, the 10th day to the phase of development of tissue granulation, and the 15th and 20th days to the phase of formation of scarring and epithelization).

Tissue slices were fixed in 4% solution of neutral formaldehyde and embedded in paraffin. Sections were stained with hematoxylin and eosin.

Statistical analysis was performed using the Student's *t* test. The Fisher's test was used to determine differences among the groups. All results are expressed as mean values ± SEM. $p<0.05$ is considered to be statistically significant.

3. RESULTS

The data on the wound-healing effect of Taurepar in rats are shown in Table 1. The wound-healing activity of the substance tested was compared with that of the well-known wound-healing drug Solcoseryl (Solco, Switzerland). The obtained results testified high efficacy of Taurepar. By the 20th day the area of wound in the group with Taurepar application was 0.9±0.3%, in the control group 8.5±1.8%, in the placebo group 7.5±1.5%, and in the group with Solcoseryl 4.0±0.8%.

Taurepar decreased the average time of complete healing of the wound in the rats up to 20th day and Solcoseryl to the 26th day. The healing in the “placebo” group was the 31st day and in the control group the 34th day (Table 2).

Daily observation indicated that the increase in the animals’ weight was statistically significant in all groups by the 15th day of the experiment. No statistically significant differences were observed among Taurepar, Solcoseryl, placebo, and control groups.

A complex of the morphological parameters describing the process of tissues regeneration was used to make a comparative evaluation of Taurepar effect on wound healing. In all groups typical processes of wound healing were observed in all skin layers. By the 20th day, sclerotization of the wound bottom, incomplete or complete epithelization and cleaning of the wound from scrab were observed. In spite of general similarities, some essential differences between the experimental groups treated with 5% Taurepar ointment and 20% Solcoseryl gel, as well as between these groups and control animals were observed.

The differences were related to (1) terms and character of cleaning of a wound from a scab, (2) terms and quality of development of regenerated connective tissue and epidermis, as well as a level of their differentiation; and (3) degree of inflammatory process expressiveness and disorder of blood circulation during regeneration.

Table 1. Effect of Taurepar on the wound area in rats (%)

Group	Before treatment	5 days	10 days	15 days	20 days	25 days
Control	100	94.8±7.3	38.9±1.8	16.8±1.5	8.5±1.8	2.8±0.8
Placebo	100	117.7±9.2	31.0±4.8	15.0±1.2	7.5±1.5	1.8±1.0
Taurepar 5% ointment	100	108.7±7.4	28.9±1.1*	10.3±1.2*	0.9±0.3*	0*
Solcoseryl 20% gel	100	89.2±10.4	22.6±6.4*	10.4±1.4*	4.0±0.8*	0.5±0.3*

* Difference is significant in relation to control by $p<0.05$.

Table 2. Effect of Taurepar on average term of complete healing of the wound in rats

Group	Period of epithelization (days)
Control	33.8 ± 1.6
Placebo	30.9 ± 1.3
Taurepar 5% ointment	20.0 ± 0.6*
Solcoseryl 20% gel	26.5 ± 1.0

* Difference is significant in relation to control by $p<0.05$.

According to the listed parameters the process of healing proceeded better in the group of animals receiving 5% ointment Taurepar. In this group early epithelization was accompanied by the growth of granulation tissue, synchronous maturation of regenerating tissues and also by an early reduction of the inflammatory activity and disorder of blood circulation. During Taurepar treatment the epithelization process began earlier (on the 5th day or earlier) and by days 10-15 complete wound healing was observed in 60% of animals. Differentiation of connective tissue and epidermis proceeded synchronously. There was no secondary damage of epidermis and no scarring of connective tissue. The wound was healed by primary intention.

Thus, the data obtained testify the positive influence of a new taurine derivative Taurepar on regeneration process of skin wounds.

4. DISCUSSION

Healing of wounds is a complex process, involving events at various levels of organization (molecular, subcellular, cellular, tissue and organ). It results in the reparation of damage, which combines the maximal restoration of the anatomic structure with minimal functional disorders. Reparative processes in a wound are inextricably related with an inflammation and form united tissue reaction to the injury. Reparative regeneration is a stereotypic process. It has the characteristic features in various tissues and organs, but the basic mechanisms are similar to those observed during skin wound healing. As it is known, regulation of the processes of normal healing is carried out by interaction of mediators, which are synthesized by infiltrated cells (neutrophils, macrophages, lymphocytes, blood platelets), resident cells (fibroblasts, epitheliocytes, endotheliocytes of capillaries) and components of the extracellular matrix (Shekhter and Serov, 1991; Greenhalgh, 1998; Singer and Clark, 1999; Moulin *et al*., 2000).

Normally there are three phases during wound healing: initial phase of inflammation,

proliferative phase of "wound closing", and a phase of wound construction with remodeling of the extracellular matrix. All three phases are interconnected and can proceed simultaneously in various sites of damage (Singer and Clark, 1999). At the end of the process of wound healing the restoration of the structure of extracellular matrix (with or without scar formation) and epithelial cover occurs. It is necessary to note that the regeneration of tissues is based on the synthesis of nucleic acids and proteins. Therefore the metabolic type drugs with a wide spectrum of pharmacological action have to regulate this process, in particular, the sulfur-containing amino acid taurine.

Recently, it was shown that taurine and its derivatives due to their physiological and pharmacological properties were allowed to regard them as effective drugs for metabolic correction and substitution therapy in different pathological states (Huxtable, 1992; Saransaari and Oja, 2000; Lima *et al*., 2001). Due to the amino group and sulfur in the taurine molecule this amino acid is a highly active natural substance, the action of which on the functional systems of the body can be appreciated generally as adaptogenic. Taurine inhibits protein membrane phosphorylation, stabilizes membrane permeability and protects hepatocytes from the toxic influences of tetrachlormethane, suppresses lipid peroxidation (Huxtable, 1992), and protects the retina from the effects of oxidants (Lima *et al*., 2001). Moreover taurine heals wounds induced by different chemical and physical effects (Degim *et al*., 2002; Farriol *et al*., 2002; Janeke *et al*., 2003).

However, taurine possesses low lipophilic activity and does not penetrate through the blood-brain barrier. Therefore, it is necessary to introduce it in high doses (Huxtable, 1992). As a consequence of these facts, the search of taurine derivates is of great importance. The new isopropylamide derivate 2-(1-phenylethylamino)ethanesulphonic acid isopropylamide hydrochloride Taurepar (Kudryashova *et al*., 1989; Sapronov *et al.,* 1999; 2000; 2004) was produced in the Department of Neuropharmacology named after S.V Anichkov, Institute of Experimental Medicine of the Russian Academy of Medical Sciences (St.Petersburg).

The present study was performed to determine the wound-healing effect of Taurepar. Our data indicate that it has positive influence on the processes of wound healing and regeneration. The wounds were healed by primary tension. These studies were confirmed by histological findings. Early beginning of epithelization (by day 5) was observed under the action of Taurepar. The complete healing of wounds in this group was noted in 60% of animals by days 10-15. The wound-healing effect of Taurepar was compared with Solcoseryl; the well-known drug used in clinic for treatment of wounds with various genesis. The area of wound in the group with Taurepar by day 20 was 0.9%, in the control group 8.5%, in the placebo group 7.5%, and in the group with Solcoseryl 4.0%. The average period of complete healing in the groups of animals treated with Taurepar was 20 days, with Solcoseryl 26 days, with placebo 31 days, and in the control group 34 days. It was indicated that the wound-healing action of Taurepar surpasses the action of Solcoseryl by 4.4 times and the healing in the control group by 9.4 times.

We suggest that the mechanism of Taurepar wound-healing effect is complex and can be associated with its antioxidant action. A similar mechanism of Taurepar activity has been found in chronic liver pathology in rats. Taurepar was shown to decrease the malondealdehyde level in hepatic tissue, to stimulate hepatocyte regeneration, and to restore liver antioxidant functions (Khnychenko *et al*., 2002).

Thus, the wide spectrum of pharmacological activity of Taurepar makes it a promising drug purposively affecting regenerative processes in different organs and tissues.

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CARDIOPROTECTIVE EFFECT OF TAURHYTHMAN: THE EXPERIMENTAL STUDY

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1. INTRODUCTION

Metabolic and non-metabolic actions of taurine provide its physiological functions in various tissues and organs (Huxtable, 1992). Phenomena associated with taurine include different cardiotropic effects. It has antiarrhythmic, positive inotropic and hypotensive action (Birdsall, 1998). There is considerable evidence that taurine modulates several processes involved in the pathogenesis of different pathological states of the heart. The most important ones are the modulation of oxygen paradox (Franconi *et al.,* 1985) and Ca^{2+}-dependent abnormalities (Kramer *et al.,* 1981; Roysommuti *et al.,* 2003). It also influences the membrane structure and function (Hamaguchi *et al.,* 1991) and osmoregulation (Rasmusson *et al.,* 1993). It is assumed that the cytoprotective properties underlie the antiarrhythmic and ischemia-reperfusion injury protection effects of taurine (Wang *et al.,* 1992; Li *et al.,* 1996; Chahine and Feng, 1998; Takahashi *et al.,* 2003). Thus, the taurine molecule may be available for derivative synthesis on purpose of developing new cardioprotective drugs.

We have synthesized a new taurine amide derivative 2-(1-methyl-2-phenylethyl)-aminoethanesulphonic acid isopropylamide hydrochloride named Taurhythman. Previously, it was found that it influences systemic hemodynamics. It produced the dose-dependent, moderately short-term cardiopulmonary reflex in cats (Sapronov *et al.,* 1998) and induced depressive changes in arterial pressure, heartbeats rate, and peripheral resistance. These effects were accompanied by an increase in stroke volume, cardiac output and venous blood return. In the isolated perfused rabbit heart Taurhythman reduced the power and rate of heartbeats and decreased the resistance of coronary arterial vessels (unpublished data). Intravenous administration in rats induced short-term

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hypotension and negative chronotropic effects (Sapronov *et al.,* 1998). Taurhythman has protected against hypoxia in different experimental models (Sapronov *et al.,* 2004) and displayed antiaggregation properties (Kudryashova *et al.,* 1989).

The aim of the present study was to evaluate the protective effects of Taurhythman against different types of arrhythmias and occlusion-induced ischemia injury.

2. METHODS

2.1. Study of Occlusion-Induced Ischemia Injury

A total of 36 adult male Wistar rats weighing 250-300 g were used. Under ether anesthesia a left thoracotomy was performed between the fourth and fifth ribs, the pericardial tissue was removed, and the left coronary artery (LCA) was ligated at the border of the left atrial appendage according to the standard technique (Selye *et al.,* 1960). After the operation, the animals were kept under observation for 72 hours. All rats with the acute infarction were randomly divided into 2 groups. Saline (control group, n=20) or Taurhythman 20 mg/kg (n=16) were administered intraperitoneally (i.p.) 10 min, 1 and 3 hours after occlusion and then twice a day until the end of the experiment. Electrocardiograms (ECG) were recorded at standard I lead (ECCMP-H3051, Russia). The magnitude of Q and QS complex was measured 72 hours after coronary occlusion. The animals were sacrificed when the experimental protocol was ended. The hearts were excised and the ventricles removed from the remaining tissue and frozen at -20ºC. Then they were cut into four cross-sectional pieces 2 mm thick. From each piece, one 50-μm slice was prepared for histochemical determination of succinate dehydrogenase activity (Loida *et al.,* 1982). Necrotic tissue was lacking dehydrogenase activity and remained unstained. The slices were photographed and the volume of infarct size was determined by the planimetric method and expressed as percentage (%) of the volume of left ventricle.

2.2. Arrhythmia Study

2.2.1. Ischemia-Induced Arrhythmias

Thirty adult male Wistar rats weighing 250-300 g were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). A polyethylene catheter was inserted into the right jugular vein for saline or drug injections. Tracheotomy was performed and the rats were intubated with a glass cannula connected to an artificial breathing apparatus. The LCA occlusion was fulfilled as described above. The rats were randomly divided into 3 groups (n=10). Taurhythman or xylocain was administered immediately after ligation as a bolus of 10 mg/kg with the subsequent infusion of 0.5 mg/kg x min^{-1} during 30 min. The control animals received a saline bolus (0.2 ml) and infusion 0.05 ml/min. ECG was recorded at lead II and rhythm disturbances were monitored for 30 min after occlusion. The number of premature ventricular beats (PVB) and the total duration and frequency of ventricular tachycardia (VT) and ventricular fibrillation (VF) were determined.

2.2.2 Non-Ischemic Arrhythmias

Sixty adult male Wistar rats weighing 200-210g (n=10 per group) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and a polyethylene catheter was inserted into the left femoral vein for drug injections. The arrhythmias were provoked by the intravenous infusion of calcium chloride 14 mg/min or aconitine 0.01 mg/min. Taurhythman, xylocain and procainamide were administered as a bolus 10 mg/kg prior to the infusion of arrhythmogenic substances with the subsequent continued infusion of 0.5 mg/kg x·min^{-1}. In the control groups the saline bolus (0.2 ml) and infusion of 0.05 ml/min were used. ECG was monitored from the onset of the experiment until the cardiac standstill. The doses of chemicals which caused the different rhythm disturbances and led to asystolia were determined.

Statistical analysis was performed using the Student's *t* test. The Fisher's test was used to determine differences among the groups. All results are expressed as the mean±SEM. A value of $p<0.05$ is considered to be statistically significant.

All procedures performed with animals were carried out in accordance with institutional and international standards (European Community Council Declarative 86/609/EEC).

3. RESULTS

3.1. Effect of Taurhythman on the Ischemic Injury

Seventy-two hours after LCA occlusion ECG of animals had specific modifications. One of them is appearance of the Q wave and formation of a pathological QS complex, which are absent in normal rats ECG (Normann *et al.,* 1961). They indicate the presence and the volume of anterior myocardial transmural necrosis and subsequent scar formation. Taurhythman decreased the Q and QS amplitudes (Table 1). Its therapeutic administration led to a decrease by 25% in the myocardial necrosis volume.

3.2 Antiarrhythmic Effect of Taurhythman

3.2.1. Effect of Taurhythman on the Ischemia-Induced Arrhythmias

The data on the protective effect of Taurhythman on the development of early occlusion arrhythmias in rats are shown in Table 2. Its antiarrhythmic activity was compared with the well-known antiarrhythmic drug xylocain. In the control animals, the early occlusion arrhythmias began 4-5 min after the LCA ligation and lasted for 20 min. All untreated rats exhibited PVB, VT and VF. As a rule, the period of rhythm disturbances was followed by restoration of the sinus rhythm.

The bolus (10 mg/kg) and subsequent infusion (0.05 mg/kg·x min^{-1}) of Taurhythman immediately after ligation was accompanied by a considerable reduction in the parameters of rhythm disorders (Table 2). Taurhythman decreased the number of PVB three times and reduced the duration of VT 16.7 times as compared with control. It completely abolished the VF. The protective effect of Taurhythman is comparable with that of xylocain.

Taurhythman also significantly decreased the incidence of VT and VF almost to the same degree when Xylocain was administered at the similar scheme and doses (Table 3).

Table 1. Effect of Taurhythman on the volume of myocardial necrosis and amplitude of Q wave and QS complex 72 hours after LCA occlusion in rats

Group	Necrosis volume (%)	Q amplitude (mV)	QS amplitude (mV)
Control	28.5 ± 1.1	0.15 ± 0.02	0.18 ± 0.03
Taurhythman	21.2 ± 1.4*	0.09 ± 0.01*	0.10 ± 0.01*

*p<0.05 compared to control, n=16-20.

Table 2. Effect of Taurhythman (bolus 10 mg/kg + infusion 0.5 mg/kg x·min^{-1}) on the development of early occlusion arrhythmias in rats with left coronary arterial ligation

Group	Premature ventricular beats (n)	Ventricular tachycardia (s)	Ventricular fibrillation (s)
Control	523 ± 26	184 ± 16	25 ± 4
Taurhythman	171 ± 12*	11 ± 5*	0
Xylocain	134 ± 9*	0	0

* p<0.05 compared to control, n=10.

Table 3. Effect of Taurhythman on the incidence of early occlusion arrhythmias in rats with LKA ligation

Group	Premature ventricular beats (%)	Ventricular tachycardia (%)	Ventricular fibrillation (%)
Control	100	100	80
Taurhythman	100	20*	0
Xylocain	100	0	0

* p<0.05 compared to control, n=10.

3.2.2 Effect of Taurhythman on the Arrhythmias Induced by Calcium Chloride

Infusion of calcium chloride 14 mg/min resulted in the development of bradyarrhythmia with extension of the P-Q interval and origin of atrioventricular (AV) blockade. These rhythm disturbances were followed by the arrest of sinoatrial (SA) node, appearance of substituted rhythms, and development of ventricular flutter and ventricular fibrillation. Flutter and fibrillation were converted to each other and were noted in 80% of the control animals. Finally, calcium chloride infusion resulted in asystolia. Taurhythman bolus of 10 mg/kg prior to the infusion of calcium chloride with the subsequent continued infusion of 0.05 mg/kg x·min^{-1} together with the arrhythmogenic substance reduced the incidence of ventricular flutter and ventricular fibrillation up to 40%. This action is comparable with that of xylocain (30%). Taurhythman and xylocain increased the arrhythmogenic doses of calcium chloride, which provoked different rhythm disturbances (Table 4).

3.2.3 Effect of Taurhythman on the Arrhythmias Induced by Aconitine

Intravenous infusion of aconitine provoked subventricular tachycardia and premature atrial beats 1-2 min after the onset of infusion. Further ventricular rhythm disturbances were observed. PVB, VT and VF were followed by asystolia. Taurhythman reduced the incidence of subventricular and ventricular arrhythmias (Table 5). In this experiment Taurhythman increased the arrhythmogenic dose of aconitine (Table 6). The protective effect of Taurhythman can be compared with the antiarrythmic action of procainamide.

Table 4. Calcium chloride doses (mg/kg) provoking different rhythm disturbances in rats

Rhythm disturbances	Control	Taurhythman	Xylocain
Bradyarrhythmia AV blockade	111 ± 10	264 ± 11*	237 ± 16*
Ventricular flutter Ventricular fibrillation	436 ± 18	495 ± 12*	504 ± 10*
Asystolia	481 ± 21	529 ± 20	536 ± 20

p<0.05 compared to control, n=10.

Table 5. Effect of Taurhythman (bolus 10 mg/kg + infusion 0.5 mg/kg x·min^{-1}) on the incidence of various rhythm disturbances provoked by aconitine

Rhythm disturbances	Control, %	Taurhythman, %	Procainamide, %
Subventricular tachycardia	80	30*	40*
Premature atrial beats	90	50*	40*
Premature ventricular beats	100	50*	60*
Ventricular tachycardia	100	60*	70*
Ventricular flutter	100	60*	60*
Ventricular fibrillation	100	50*	40*

* p<0.05 compared to control, n=10.

Table 6. Aconitine doses (μg/rat) provoking different rhythm disturbances in rats

Rhythm disturbances	Control	Taurhythman	Procainamide
Subventricular tachycardia	27 ± 2	51 ± 3*	48 ± 2*
Premature atria beats	39 ± 2	75 ± 4*	72 ± 3*
Premature ventricular beats	46 ± 2	79 ± 3*	87 ± 3*
Ventricular tachycardia	64 ± 5	117 ± 8*	137 ± 9*
Ventricular flutter	84 ± 9	134 ± 9*	159 ± 10*
Ventricular fibrillation	139 ± 10	164 ± 10	174 ± 12
Asystolia	192 ± 18	245 ± 19	276 ± 21

* p<0.05 compared to control, n=10.

4. DISCUSSION

The action of taurine at the heart level has been studied for several tens of years and the principal mechanisms of its cardiovascular effects have been elaborated (Chapman *et al.,* 1993). The decrease in myocardial damage during ischemia caused by taurine is associated mainly with membrane stabilization. It was reported that this property is due to the capacity of taurine to scavenge several species of free radicals, which are produced during ischemia and lead to membrane phospholipid peroxidation (Chahine and Feng, 1998; Hanna *et al.,* 2004). As a result, preserved membrane fluidity, secretory functions and transmembrane ionic gradients prevent the enhancement of membrane Ca^{2+} permeability and cell Ca^{2+} overload, increase the K^+ concentration in cardiomyocytes and regulate Na^+ transport (Satoh and Sperelakis, 1998). These effects determine the positive role of taurine in the prevention of ischemia injury. This makes taurine and its derivatives available in the development of new drugs for cardiology.

In order to enhance the pharmacological efficiency of taurine, its amide derivative 2-(1-methyl-2-phenylethyl)aminoethanesulphonic acid isopropylamide hydrochloride Taurhythman was synthesized. The present study has been performed to determine its protective effects against different types of arrhythmias and ischemia injury. Our data

indicate that Taurhythman can reduce the terminal necrosis volume after LCA occlusion by 25% and diminish the Q wave amplitude 1.7 times. The simultaneous decrease in the incident and amplitude of the pathological QS complex was observed.

Taurhythman possesses the most evident antiarrhythmic effect. In the model of ischemia-induced arrhythmias it decreased the number of premature ventricular beats and duration of ventricular tachycardia by 67% and 94%, correspondingly. The VT incident was lowered to 20% as compared with 100% in control. Taurhythman exhibited antifibrillatory activity, completely preventing fibrillation in all treated rats. The antiarrhythmic effect of Taurhythman was similar to that of xylocain, the well-known antyarrhythmic drug used in clinic to arrest ventricular rhythm disturbances in the acute coronary deficiency. The doses and treatment scheme for Taurhythman were the same as recommended for xylocain. Development of early occlusion arrhythmias in rats is a two-phase process (Ogawa *et al.,* 1979). The first 1a phase lasts for about 10 min after occlusion and arises from the activation of re-entry mechanism. The second one (1b phase) has its peak at 15-20 min. It is considered to result from catecholamine release, activation of latent centers and triggering activity. In the present experiments, two phases of arrhythmias were also obtained. Xylocain completely abolished 1b phase and markedly reduced 1a phase. Taurhythman had the same action, suppressing the re-entry mechanism and preventing trigger activity.

Furthermore Taurhythman showed an apparent positive influence on the rhythm disturbances produced by chemicals.

The increase in Ca^{2+} during the infusion of calcium chloride results in heterogeneous conductance, sinoatrial blockade and disturbances in atrial, atrioventricular and ventricular conductance. Later, the re-entry mechanism generates ectopic impulses, which become the reason of ventricular tachycardia, flutter and fibrillation. Taurhythman decreased two times the incident of ventricular flutter and fibrillation and reduced the calcium chloride doses responsible for the development of all types of rhythm disturbances.

The pathogenetic mechanism of aconitine-induced arrhythmias involves activation of potential-dependent Na^+ channels and inhibition of their inactivation (Tanz, 1974). The increase in the intracellular Na^+ concentration causes Ca^{2+} influx in cardiomyocytes. It results in cell membrane depolarization, hyperexcitability, increase in automaticity and development of trigger activity. In this case, the antiarrhythmic activity of Taurhythman is comparable with that of procainamide, which inhibits membrane penetration of Na^+ ions. It was shown that Taurhythman decreased nearly two times the incident of all types of rhythm disturbances caused by aconitine and increased its arrhythmogenic doses.

Thus, the obtained data testify the antiarrhythmic properties of the new taurine amide derivative Taurhythman. Like taurine, it has amino and sulfonic groups, which play an important role in the prevention of arrhythmias (Welty *et al.,* 1997). When compared with taurine, Taurhythman exhibited this activity in doses of 10-20 mg/kg, i.e. ten times less than taurine (Birdsall, 1998). Probably the antiarrhythmic effect of Taurhythman became stronger due to the addition of one more amino group and phenyl ring. The presence of amino groups and phenyl ring is the common feature in the molecular structures of Taurhythman, xylocain, and procainamide.

The use of arrhythmia models with different mechanisms showed that antiarrhythmic activity of Taurhytman is probably connected with the maintenance of ionic homeostasis. We propose that its membrane stabilizing action can provide this effect. It has been shown previously that Taurhythman decreases to 70% Ca^{2+} fluxes in neurons *in vitro* and

prevents the decrease in membrane-bound Na^+,K^+-ATPase activity (unpublished data). Anti-ischemic and antyarrhythmic action of Taurhythman, together with other cardiovascular effects, and antihypoxic and antiaggregation properties points out that Taurhythman can be a promising cardioprotective drug.

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NEUROPROTECTIVE EFFECT OF A NEW TAURINAMIDE DERIVATIVE — TAUREPAR

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1. INTRODUCTION

One of the important directions of modern pharmacology is the development of new drugs promoting the decrease of neurodegenerative process in the central nervous system (CNS) diseases, which pathogenesis includes ischemia. Insult and traumatic injury of the spinal cord are among them.

Ischemia is known to induce a complex of pathobiochemical changes leading to damage and finally to necrotic destruction of neurons (Borshenko *et al.,* 2000; Gusev and Skvortsova, 2001). Energy metabolism disorder and concomitant activation of free radical lipid peroxidation triggers these processes.

Considering the important role of ischemia in pathogenesis of the above-mentioned diseases, inclusion in their complex therapy the drugs, capable of metabolic protection, and prevention of secondary neuron ischemic damage, might be expedient. This approach can limit the expansion of the initial necrotic area. Antihypoxants and antioxidants are the promising preparations possessing neuroprotective action. Among such compounds is 2-(1-phenylethyl)aminoethanesulphonic acid isopropylamide hydrochloride (Taurepar) synthesized on the basis of taurine in the Department of Neuropharmacology of the Institute of Experimental Medicine of the Russian Academy of Medical Sciences.

Taurine is known to display antihypoxic and marked antioxidant properties (Mankovskaya *et al.,* 2000; Bidri and Choay, 2003). Taurepar also shows antihypoxic properties. Previously it was demonstrated that it stimulates energy metabolism and antioxidant system in myocardial ischemia (Khnychenko *et al.,* 2001) and toxic liver damage (Khnychenko *et al.,* 2002).

The aim of the present study was to estimate neuroprotective effect of Taurepar in experimental cerebral ischemia and spinal cord compression injury. Piracetam and cerebrolysin were used as reference compounds.

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2. METHODS

2.1. Model of Total Cerebral Ischemia

Experiments were carried out on 160 Wistar male rats with body weight of 180-200 g. Under ether anesthesia bilateral occlusion of common carotid arteries (BOCCA) was done. All animals were assigned to 4 groups (n=40). The sham-operated rats were exposed to all procedures except tying up a surgical thread; the treated rats after BOCCA received Taurepar 25 mg/kg intraperitoneally (i.p.) or piracetam 100 mg/kg i.p., and the control rats were given the same volume of saline. The initial dose was given 30 min after occlusion and then two times daily for all period of the experiment. Ten animals from each group were observed for 14 days. Survival rate, survival rate median, and dynamics of relative drug efficiency were determined. Survival median is a time point when 50% of animals remain alive. Relative drug efficiency was calculated as $RE = (D - K)\cdot(1 - K)^{-1}$, where RE is the relative drug efficiency, D the part of the survived animals treated with the drug, and K the part of the survived animals at the same time in the control group (Ivanova and Bobkov, 1984). The remaining animals were sacrificed 1.5, 24 and 72 hours after BOCCA (n=10 in each group). The cerebral hemispheres were excised, put in liquid nitrogen, and stored there at -195ºC for determinations of lactate, pyruvate, lipid hydroperoxides (LHP), malone dialdehyde (MDA), and superoxide dismutase (SOD) and catalase activity spectrophometrically as previously described (Johansson and Borg, 1988; Bulion *et al.,* 2003). The pyruvate/lactate ratio was used to assess the energy metabolism.

2.2 Model of Spinal Cord Compression Trauma

These experiments were carried out on 300 Wistar male rats with body weight of 180-200 g. Under ether anesthesia compression was induced at the low thoracic level (Th_{10}-Th_{11}) using the method previously described (Bulion *et al.,* 2005a). The animals were placed in stereotaxic device and the metal rod (length 7 cm, diameter 3 mm) was fixed in it. To produce compression the rod was lowered 5 mm below the level of the dura mater. Compression time was 1 min. Preliminary experiments have shown that this impact causes paraplegia of the hind limbs. All rats were assigned to 4 groups (n=80-100): the sham-operated animals were without compression, the rats after compression were given Taurepar (25 mg/kg) or cerebrolysin (1 ml/kg) i.p., and the control rats received the same volume of saline. The initial dose was given 30 min after compression. Later on, Taurepar two times a day and cerebrolysin once a day were introduced up to the end of the experiment. Thirty animals from each group were observed for 14 days. Dynamics of survival rate, survival rate median, and RE were estimated as described above. 1, 3, 7, and 14 days after trauma a degree of hind limbs motor function was estimated using 4-point scale: 1 point – normal, 2 points – moderate paraparesis, 3 points – marked paraparesis, 4 points – paraplegia (Sufianova *et al.,* 2002). At 1, 3, 7, and 14 days after trauma 18-20 animals from each group were anesthetized with ether and liquor samples were taken from cisternae subarachnoidales for lactate, pyruvate, MDA, and redox glutathione (RG) content, and SOD activity determinations (Bulion *et al.,* 2003).

Statistical analysis was performed using the Student's *t* test. The Fisher's test was used to determine differences among the groups. All results are expressed as the mean ± SEM. A value of $p<0.05$ is considered to be statistically significant.

3. RESULTS

3.1. Neuroprotective Effects of Taurepar and Piracetam in Total Cerebral Ischemia

BOCCA lasting for 1.5 h resulted in the increase in the lactate content by 160% and decrease in the pyruvate content by 42 % in cerebral tissue in comparison with the sham-operated animals (Table 1). The pyruvate/lactate ratio increased from 9.8 to 44.3. Later on (24 h and 72 h) it diminished up to 22.2 and 13.2, respectively. Taurepar and piracetam prevented the increase in the lactate and the decrease in the pyruvate levels during the whole experiment. However, at the early term (1.5 h) the effect of Taurepar was slightly greater than that of piracetam.

In the control group 1.5 h after BOCCA, the LHP and MDA levels were increased by 37% and 35%, and 24 h later by 42% and 89%, respectively (Table 2). At the same time the SOD and catalase activities decreased by 26% and 22% in 1.5 h and by 35% and 31% in 24 h, correspondingly. After 72 h, the LHP intensity and antioxidant system (AOS) enzyme activities began normalize but did not reach the level of sham-operated rats. Taurepar maintained the LHP product content and AOS activity at the normal level from 1.5 to 72 h. The highest correcting effect was noted at 24 h. Piracetam also prevented lipid peroxidation (LP) intensification and AOS depletion during all periods of observation (Table 2). However, at 24 h its protection against LP was less effective when compared with the action of Taurepar.

Taurepar reduced animal lethality from the first day of treatment and by day 14 the survival rate increased up to 40% (Fig. 1A) in comparison with 10% in controls. Piracetam increased the survival rate up to 30% by the end of experiment. However, during the first 6 days it did not influence the lethality. The median of survival rate in the control rats was 4 days, in the rats treated with Taurepar 10 days, and in the animals treated with Piracetam 4 days (Fig. 1A). The relative efficiency of Taurepar from 1 to 14 days of experiment was more significant when compared to piracetam (Fig. 1B).

Table 1. Effect of Taurepar (25 mg/kg) and piracetam (100 mg/kg) on the lactate and pyruvate levels in the rat brain after bilateral occlusion of common carotid arteries

Group	Time h	Lactate μmol/g	Pyruvate μmol/g	Lactate/pyruvate
Sham operation		2.55 ± 0.04	0.26 ± 0.02	9.8
Control	1.5	6.65 ± 0.16*	0.15 ± 0.01*	44.3
	24	3.99 ± 0.08*	0.18 ± 0.01*	22.2
	72	2.24 ± 0.04	0.17 ± 0.01*	13.2
Taurepar	1.5	2.73 ± 0.08#	0.21 ± 0.02#	13.0
	24	2.34 ± 0.05#	0.25 ± 0.02#	9.4
	72	2.27 ± 0.06	0.25 ± 0.03#	9.1
Piracetam	1.5	3.47 ± 0.07#	0.20 ± 0.02#	17.3
	24	2.41 ± 0.04#	0.25 ± 0.03#	9.6
	72	2.32 ± 0.11	0.25 ± 0.03#	9.3

* $p<0.05$, compared to sham operation; # $p<0.05$, compared to control.

Table 2. Effect of Taurepar (25 mg/kg) and piracetam (100 mg/kg) on lipid peroxidation and the antioxidant system in the rat brain after bilateral occlusion of common carotid arteries

Group	Time h	LHP OD_{480}	MDA nmol/g	SOD Activity/mg protein	Catalase μmol H_2O_2/mg protein
Sham operation		0.097 ± 0.004	7.39 ± 0.31	3.48 ± 0.12	7.35 ± 0.31
Control	1.5	0.133 ± 0.002*	9.98 ± 0.19*	2.57 ± 0.21*	5.73 ± 0.27*
	24	0.138 ± 0.007*	13.97 ± 0.36*	2.26 ± 0.05*	5.07 ± 0.21*
	72	0.121 ± 0.004*	10.35 ± 0.43*	2.89 ± 0.12*	5.88 ± 0.21*
Taurepar	1.5	0.102 ± 0.006#	7.48 ± 0.45#	3.57 ± 0.28#	7.28 ± 0.18#
	24	0.085 ± 0.003#	5.03 ± 0.40#	3.19 ± 0.13#	7.00 ± 0.26#
	72	0.094 ± 0.001#	7.11 ± 0.26#	3.58 ± 0.09#	7.45 ± 0.21#
Piracetam	1.5	0.112 ± 0.003#	8.18 ± 0.25#	3.37 ± 0.18#	6.93 ± 0.26#
	24	0.098 ± 0.005#	7.26 ± 0.67#	3.12 ± 0.10#	6.84 ± 0.24#
	72	0.098 ± 0.004#	7.38 ± 0.47#	3.32 ± 0.09#	6.88 ± 0.25#

*$p<0.05$, compared to sham operation; #$p<0.05$, compared to control.

3.2. Neuroprotective Effects of Taurepar and Cerebrolysin in Spinal Cord Compression Trauma

Spinal cord compression trauma resulted in significant damage in energy metabolism of the spinal cord (Table 3). One day after trauma the lactate level in the control animal liquor increased by 230% and the pyruvate content decreased by 69%. The lactate/pyruvate ratio increased from 9.0 up to 95.5. On more remote terms of trauma (3 and 7 days) the anaerobic glycolysis intensity decreased but remained much higher than in the sham-operated rats. By day 10 all control animals died and biochemical analyses could not be done.

Taurepar reduced the lactate content and increased the pyruvate content from the first day of treatment, restoring their basal levels by day 14 (Table 3). Cerebrolysin was significantly less effective than Taurepar.

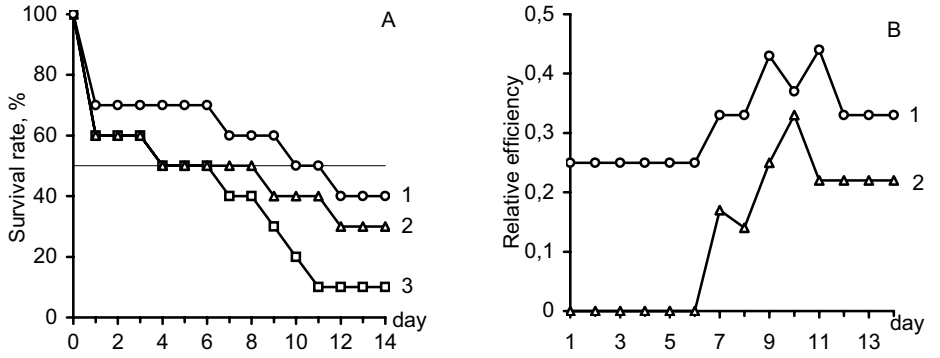

Figure 1. Effect of Taurepar and piracetam on the dynamics of survival rate and survival rate median (A); and relative drug efficiency (B) in the rats with bilateral occlusion of common carotid arteries. 1-Taurepar 25 mg/kg i.p., 2-Piracetam 100 mg/kg i.p., 3-control (saline). See text for further explanation.

Table 3. Effect of Taurepar (25 mg/kg) and cerebrolysin (1 mg/kg) on the lactate and pyruvate levels in rat liquor after spinal cord compression trauma

Group	Time day	Lactate μmol/g	Pyruvate μmol/g	Lactate/pyruvate
Sham operation		1.72 ± 0.03	0.19 ± 0.01	9.0
Control	1	5.73 ± 0.08*	0.06 ± 0.01*	95.5
	3	4.61 ± 0.21*	0.09 ± 0.01*	51.2
	7	3.88 ± 0.12*	0.13 ± 0.02*	29.8
	14	all	animals	died
Taurepar	1	3.11 ± 0.08#	0.09 ± 0.01#	34.5
	3	2.93 ± 0.05#	0.13 ± 0.01#	22.5
	7	2.04 ± 0.03#	0.14 ± 0.01	14.6
	14	1.71 ± 0.03#	0.17 ± 0.01#	10.0
Cerebrolysin	1	4.92 ± 0.12#	0.06 ± 0.01	82.0
	3	3.54 ± 0.12#	0.10 ± 0.01	35.4
	7	2.46 ± 0.07#	0.11 ± 0.01	22.4
	14	2.09 ± 0.10#	0.13 ± 0.01	16.1

* $p<0.05$, compared to sham operation; # $p<0.05$, compared to control.

Table 4. Effect of Taurepar (25 mg/kg) and cerebrolysin (1 mg/kg) on the lipid peroxidation and antioxidant system during spinal cord compression trauma

Group	Time day	MDA nmol/g	SOD Activty/mg protein	Reduced glutathion μmol/g
Sham operation		4.19 ± 0.05	0.56 ± 0.03	1.32 ± 0.08
Control	1	7.47 ± 0.17*	0.22 ± 0.02*	0.48 ± 0.05*
	3	5.82 ± 0.10*	0.31 ± 0.03*	0.63 ± 0.04*
	7	5.16 ± 0.19*	0.46 ± 0.03*	0.80 ± 0.04*
	14	all	animals	died
Taurepar	1	5.11 ± 0.10#	0.39 ± 0.03#	0.60 ± 0.01#
	3	4.28 ± 0.07#	0.46 ± 0.03#	1.03 ± 0.08#
	7	4.16 ± 0.03#	0.50 ± 0.02	1.20 ± 0.03#
	14	4.08 ± 0.07#	0.53 ± 0.01#	1.25 ± 0.03#
Cerebrolysin	1	6.61 ± 0.14#	0.31 ± 0.02#	0.51 ± 0.02
	3	4.97 ± 0.09#	0.36 ± 0.01	0.65 ± 0.04
	7	4.61 ± 0.19#	0.41 ± 0.02	0.89 ± 0.02
	14	4.41 ± 0.13#	0.49 ± 0.02	1.20 ± 0.02#

* $p<0.05$, compared to sham operation; # $p<0.05$, compared to control.

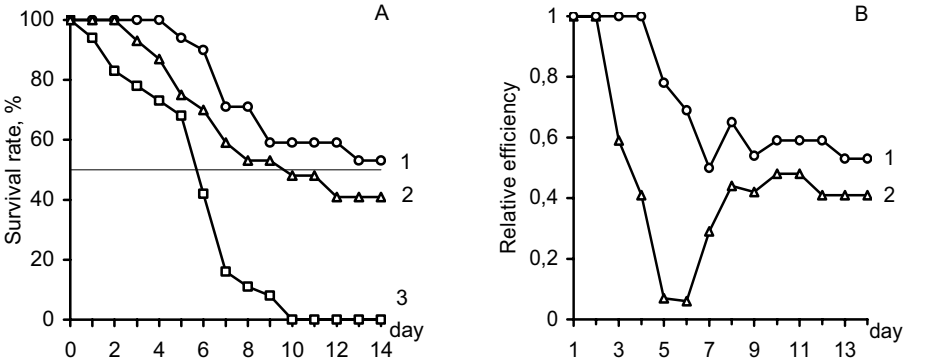

Figure 2. Effect of Taurepar and cerebrolysin on the dynamics of survival rate and survival rate median (A); relative drug efficiency (B) in the rats with spinal cord compression trauma. 1–Taurepar 25 mg/kg i.p., 2-Cerebrolysin 1 ml/kg i.p., 3-control (saline). See text for further explanation.

On the first day of trauma, the MDA level in liquor of the control animals increased by 78%, SOD activity decreased by 61%, and RG content was reduced by 64%. On days 3 and 7 after trauma, the MDA content decreased, while AOS enzyme activities increased. However, the levels of these parameters differed considerably from those of the sham-operated animals.

Taurepar reduced MDA production on the first day after compression. On day 3 it was already at the normal level. It restored AOS activity up to the basal level on day 7 (Table 4). The cerebrolysin-treated rats exhibited reduced LP intensity and reactivated AOS system only on day 14.

Paraplegia (4 points) of the hind limbs was noted in all rats with compressive injury. Taurepar treatment reduced the manifestation of neurological disorder up to 3.20±0.18, 2.87±0.25, 2.04±0.36, and 1.40±0.22 points on days 1, 3, 7 and 14, respectively ($p<0.001$). Cerebrolysin also restored motor functions: the degree of neurological disorder reduced to 2.93±0.19, 2.80±0.26, 2.14±0.36 and 1.75±0.24 points, respectively ($p<0.001$).

In the control group compressive spinal cord damage resulted in 100% death of the animals towards day 10 after trauma (Fig. 2A). Taurepar considerably increased the lifetime of rats with the injured spinal cord and by the end of observation period (14 days) 53% of treated animals survived. Cerebrolysin increased the survival rate of rats by day 14 up to 41%. The survival rate median in the control rats was 5.5 days, in the rats receiving cerebrolysin 9.5 days, while in the rats treated with Taurepar it was more than 14 days (Fig. 2A). Relative efficiency of both compounds (Fig. 2B) was similar during the first two days of treatment. Within the next 12 days, the effect of Taurepar exceeded 1.2-11.5 times the effect of cerebrolysin, especially during the first week after compression.

4. DISCUSSION

The results of the present study showed that in BOCCA Taurepar produced a protective effect on energy metabolism, prevented LP hyperactivation, and AOS inhibition. These positive effects were more evident than after piracetam treatment. It is significant that Taurepar preserves the activity of AOS at the beginning of ischemic injury (1.5 h). This fact supports the rapid involvement of Taurepar in the processes of hydroxyl radical formation at a stage of LP initiation. Previously, it was reported that Taurepar activates the glutathione system of AOS (Khnychenko *et al.,* 2001).

The results of the present study demonstrate that Taurepar is more effective than piracetam in reduction of lethality in the rats with cerebral ischemia and its relative efficiency is much greater than that of piracetam. Taurepar increased the survival rate from the first day of treatment, whereas piracetam began to show this effect only from day 7. Such delayed action is probably connected with potentiation of the 2-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) subtype of glutamate receptors, increasing the glutamate toxic effect at early terms of ischemia (Copani *et al.,* 1992). Moreover, the anti-ischemic activity of piracetam is known to be associated with its ability to stimulate aerobic and anaerobic glucose oxidation and to suppress LP in mitochondria (Voronina and Seredenin, 1998). These effects can be completely realized only after stabilization of hemodynamics and increased glucose supply of the brain. Our previous studies showed that Taurepar is a preparation of a metabolic type of action with cytoprotective effect (Khnychenko *et al.,* 2001, 2002). It is known that therapeutically more effective neuroprotective compounds are those substances which simultaneously stimulate metabolic processes and improve cerebral blood supply (Gannushkina *et al.,* 1998; Bulion *et al.,* 2004, 2005b). The higher relative efficiency of Taurepar may result from its ability to influence cerebral blood circulation along with metabolic protection.

In spinal cord compression trauma the positive effect of Taurepar was more significant than that of cerebrolysin at all terms. Taurepar reduced LP intensity and restored AOS activity up to the initial level on day 7 of treatment, while cerebrolysin did it on day 14. Taurepar and cerebrolysin promoted restoration of the abnormal motor function of the hind limbs of rats. The degree of manifestation of this neurological disorder began to decrease from the first day of treatment. Taurepar significantly increased the survival rate in animals with spinal cord compression trauma and was obviously more effective than cerebrolysin. The relative efficiency of Taurepar was greater than that of cerebrolysin. It is possible that this action, as well as in cerebral ischemia, is due to the ability of Taurepar to produce vasoactive effect alongside with metabolic protection. It was reported that cerebrolysin has no such combined activity. It activates metabolic processes and nervous activity of the brain, but does not improve its blood supply, which results in a more severe course of ischemia in its acute period (Gannushkina *et al.,* 1998).

Thus, the results of the present study show that Taurepar produces a marked neuroprotective effect in total cerebral ischemia and in compression spinal cord trauma. It manifests itself in normalization of the processes of energy formation, LP decrease, and AOS reactivation, in restoration of the hind limb functions and in the considerable reduction of lethality. The neuroprotective action of Taurepar is more discernible than those of piracetam and cerebrolysin. The data obtained imply that Taurepar is a potential drug for metabolic protection in cerebral ischemia and compressive spinal cord trauma.

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INDEX