Investigation of the Role of a Supplementation with Taurine on the Effects of Hypoglycemic-Hypotensive Therapy Against Diabetes-Induced Nephrotoxicity in Rats

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Abstract This study has examined the role of supplementing a treatment of diabetic rats with captopril (CAP), metformin (MET) or CAP-MET with the antioxidant amino acid taurine (TAU) on biochemical indices of diabetes-induced metabolic changes, oxidative stress and nephropathy. To this end, groups of 6 male Sprague-Dawley rats (250–375 g) were made diabetic with a single, 60 mg/kg, intraperitoneal dose of streptozotocin (STZ) in 10 mM citrate buffer pH 4.5 and, after 14 days, treated daily for up to 42 days with either a single oral dose of CAP (0.15 mM/kg), MET (2.4 mM/kg) or TAU (2.4 mM/kg), or with a binary or tertiary combination of these agents. Rats receiving only 10 mM citrate buffer pH 4.5 or only STZ served as negative and positive controls, respectively. All rats were sacrificed by decapitation on day 57 and their blood and kidneys collected. In addition, a 24 h urine sample was collected starting on day 56. Compared to normal rats, untreated diabetic ones exhibited frank hyperglycemia (+313%), hypoinsulinemia (−76%) and elevation of the glycated hemoglobin value (HbA_{1c}, +207%). Also they showed increased plasma levels of Na^+ (+35%), K^+ (+56%), creatinine (+232%), urea nitrogen (+158%), total protein (−53%) and transforming growth factor-β1 (TGF-β1, 12.4-fold) values. These changes were accompanied by increases in the renal levels of malondialdehyde (MDA, +42%), by decreases in the renal glutathione redox state (−71%), and activities of catalase (−70%), glutathione peroxidase (−71%) and superoxide dismutase (−85%), and by moderate decreases of the urine Na^+ (−33%) and K^+ (−39%) values. Following monotherapy, MET generally showed a greater attenuating effect than CAP or TAU on the changes in circulating glucose, insulin and HbA_{1c} levels, urine total protein, and renal SOD activity; and CAP appeared more potent than TAU and MET, in that order, in antagonizing the changes in plasma creatinine and urea nitrogen levels. On the other hand, TAU generally provided a greater protection against changes in glutathione redox state and in CAT and GPx activities, with other actions falling in potency between those of CAP and MET. Adding TAU to a treat-

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ment with CAP, but not to one with MET, led to an increase in protective action relative to a treatment with drug alone. On the other hand, the actions of CAP-MET, which were about equipotent with those of MET, became enhanced in the presence of TAU, particularly against the changes of the glutathione redox state and activities of antioxidant enzymes. In short, the present results suggest that the addition of TAU to a treatment of diabetes with CAP or CAP-MET, and sometimes to one with MET, will lead to a gain in protective potency against changes in indices of glucose metabolism and of renal functional impairment and oxidative stress.

Keywords Diabetic nephropathy • Streptozotocin • Captopril • Metformin Taurine • Insulin • Blood and plasma biochemical parameters • Renal function tests Oxidative stress • Histological changes

Abbreviations

CAP	Captopril
CAT	Catalase
CRN	Creatinine
DM	Diabetes mellitus
DN	Diabetes nephropathy
GLC	Glucose
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Glutathione disulfide
HbA_{1c}	Glycated hemoglobin
INS	Insulin
MDA	Malondialdehyde
MET	Metformin
SOD	Superoxide dismutase
STZ	Streptozotocin
TAU	Taurine
TGF-β1	Transforming growth factor β1
UN	Urea nitrogen

1 Introduction

Diabetic nephropathy (DN) is as an important complication of diabetes mellitus (DM) affecting approximately one-third of patients with insulin-dependent diabetes and representing a major driving force of end-stage renal failure and mortality (Barnes and Viberti 1994). This functional disorder of diabetes has been well studied

in patients with types 1 and 2 DM, and it has been found characterized by microalbuminuria, renal hyperfiltration, and increased permeability to blood urea and to macromolecules, which are typically seen after 5 years of type 1 DM (Butt et al. 2010) and in about 7–10 years of type 2 DM (Barnes and Viberti 1994; Satirapoj and Adler 2014). While a persistent microalbuminuria is associated with an increased risk of developing cardiovascular disease and with progression to renal disease, values in excess of 300 mg/day of albuminuria (macroalbuminuria) are considered to represent overt nephropathy. At this point, the rate of loss of glomerular filtration rate and the development of hypertension are common to both type 1 and type 2 DM (Satirapoj and Adler 2014), and the introduction of renal replacement therapy in the form of dialysis or transplantation is not a rare event (Barnes and Viberti 1994). Also, the chronic hyperglycemic state of diabetes is responsible for the formation and accumulation of advanced glycated end products (AGEs) in the kidney and which, upon binding to AGEs receptors on mesangial cells, become major contributors to the development and progression of the glomerular and tubular structural changes that are seen in DN, including glomerular basement membrane thickening, glomerulosclerosis and tubulointerstitial fibrosis (Goh and Cooper 2008). There is also glomerular mesangial expansion as a result of initial cell proliferation and cell hypertrophy brought about at the beginning by mesangial stretch and pressure and by high glucose levels, and later by transforming growth factor-β1 (TGF-β1) and fibrosis (Butt et al. 2010). As a consequence, dysfunction of the mesangium, glomerular capillary wall, tubulointerstitium and vasculature will ensue (Satirapoj and Adler 2014). In addition, biochemical changes such as activation of the polyol pathway key enzyme aldose reductase, stress-activated signaling pathways (Evans et al. 2002) and protein kinase C (Tavafi 2013), an activator of mesangial expansion, play an important role in the development of diabetic renal complications.

Hyperglycemia is known to promote oxidative stress by contributing to the formation of reactive oxygen (ROS) and nitrogen (RNS) species, and to play a major role in the pathogenesis of DN by creating a state of oxidative stress that not only causes damage to cell membranes and to intracellular enzymes but also promotes apoptotic and necrotic cell death and alters gene expression (Allen et al. 2005). In addition, there is an increase in the levels of malondialdehyde (MDA), an index of lipid peroxidation (LPO), and a deficiency of glutathione (GSH) and of antioxidant enzymes such as catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD) in the blood and kidney of both diabetic humans (Hodgkinson et al. 2003) and animals (Kędziora-Kornatowska 1999; Kędziora-Kornatowska et al. 2000).

Drugs such as metformin (MET), a biguanide derivative that is widely used in individuals with type 2 DM and for which it represents a first line oral therapy for decreasing the plasma glucose concentration, may be useful in delaying or slowing down the development of DN (Mogensen et al. 1995) especially if it is used in conjunction with lifestyle modifications such as dietary restriction, weight control and physical activity (Viollet et al. 2012). The favorable effect of this drug on hyperglycemia has been related to an ability to augment peripheral glucose uptake through an insulin-sensitizing action without stimulating insulin secretion

(DeFronzo and Goodman 1995; Seufert et al. 2004), and to reduce glucose production through inhibition of gluconeogenesis (Seufert et al. 2004) and increased nonoxidative metabolism, including the formation of glycogen (Setter et al. 2003). The antigluconeogenic effect of MET has been related to an ability to reduce hepatic energy status (Fortes et al. 2010). MET has also been found to decrease urinary albumin excretion rates and to prevent podocyte injury in a rat model of type 2 DM (Kim et al. 2012), to reduce markers of oxidative stress, to improve renal antioxidant enzyme activity, to ameliorate apoptosis and, in general, to act as a nephroprotectant in DN (Alhaider et al. 2011; Viollet et al. 2012).

Evidence is also available in support of the benefits of therapy with angiotensin converting enzyme (ACE) inhibitors since they can be useful in impeding the progress of proteinuria and in preventing the increase of urinary albumin excretion in nonhypertensive patients with INS-dependent DM and persistent microalbuminuria (Viberti et al. 1994). In general, ACE inhibitors has been found effective in limiting macrovascular complications and in reducing the progression of retinopathy, albuminuria and nephropathy in patients with type 2 DM (King et al. 1999). As a result, drugs like CAP appear to be useful to control blood pressure in hypertensive diabetic patients with nephropathy by reducing the decline in kidney function but without impairing glucose tolerance (González-Sicilia de Llamas et al. 1991).

Taurine (TAU), the sulfonic acid analogue of β-alanine, has been extensively investigated as an antioxidant both *in vitro* and *in vivo*. In spite of lacking a readily oxidizable functional group and of having a demonstrable low free radical scavenging action, this ubiquitous nonprotein amino acid has consistently been found to protect cultured cells, organs and mammalian species against the deleterious consequences of oxidative stress fostered by a myriad of chemical agents (Das and Sil 2012; Sayed et al. 2012), biochemicals (Kalaz et al. 2013), drugs (Das et al. 2012; Hagar et al. 2006; Shao et al. 2012), toxins (Bhavasar et al. 2009; Das et al. 2012) and disease states (Ito et al. 2012; Rikimaru et al. 2012). Mechanisms such as elevation of the activities of the antioxidant enzymes CAT, SOD (Vohra and Hui 2001), GPx (Anand et al. 2011; Vohra and Hui 2001) and glutathione reductase (GR) (Anand et al. 2011), preservation of the expression and secretion of extracellular SOD (Nonaka et al. 2001), protection of GSH stores (Oudit et al. 2004) and the intracellular redox status (Acharya and Lau-Cam 2013), prevention or reduction of intracellular calcium increase (Chen et al. 2001; Yamauchi-Takihara et al. 1988) and movement (Timbrell et al. 1995), membrane stabilization (Chen et al. 2001; Timbrell et al. 1995), and direct binding to reactive aldehydes (Ogasawara et al. 1993) and to free radicals (Nakamura et al. 1993), are some of the explanations that have been mentioned to account for the seemingly unexpected beneficial actions derived from this sulfur-containing compound.

Taking into account the benefits that have been demonstrated earlier from a treatment of diabetes with MET, CAP or TAU, the present study was undertaken in rats made diabetic with the diabetogen streptozotocin to compare the effects that these compounds could have on diabetes-induced metabolic and renal function alterations when used singly or as binary or ternary combinations.

2 Materials and Methods

2.1 Treatment Compounds

The treatment agents CAP, MET and TAU were obtained from Sigma-Aldrich, St. Louis, MO, USA. Streptozotocin (STZ) was purchased from A.G. Scientific, San Diego, CA, USA. All other chemicals were from Sigma-Aldrich, St. Louis, MO, USA.

2.2 Animals

Each experimental group consisted of 6 male Sprague-Dawley rats, 200–225 g in weight, obtained from Taconic Farms, Germantown, New York, USA. During the entire study, including an acclimation period of 7 days, the rats were kept in a constant temperature (23 ± 1 °C) and humidity room and on a normal 12 h light-12 h dark cycle, and had free access to a standard rodent diet and filtered tap water.

2.3 Treatment Solutions and Treatments

STZ was dissolved in 10 mM citrate buffer pH 4.5 to provide a 0.23 mM solution. Solutions of the treatment compounds CAP, MET and TAU were freshly made each day in physiological saline. Diabetes was induced with a single, 60 mg/kg/mL, intraperitoneal dose of STZ. After 14 days, the diabetic rats started to receive a daily dose of a treatment agent (0.15 mM/kg of CAP, 2.4 mM/kg of MET or TAU, singly or as binary or ternary combinations) by oral gavage. When more than one treatment agent was involved, they were provided at 15 min intervals. Control (normal) rats received a 2 mL volume of physiological saline by the oral route in place of a treatment agent solution. Body weights and tail vein blood glucose levels were monitored on a weekly basis for a total of 56 days.

2.4 Sampling and Samples

A drop of blood was collected each week from the tail vein and used to measure the blood glucose level with the help of a commercial glucometer (TRUEtrack™ and test strips, both from Nipro Diagnostics, Fort Lauderdale, FL, USA). Only rats exhibiting a blood glucose level above 250 mg/dL were used in the study. A 24 h urine sample was collected from each rat in a metabolic cage from days 56 to 57. On day 57, the rats

were sacrificed by decapitation to collect their bloods in heparinized tubes. Immediately thereafter the kidneys were surgically excised with the help of a scalpel and kept on ice until needed for biochemical assays. A portion of each blood sample was used to measure the level of glycated hemoglobin and the other portion was centrifuged at 700 × g for 10 min to obtain the plasma fraction. A representative portion of kidney, dried with a piece of filter paper and weighing about 500 mg, was cut into small pieces with a razor blade, mixed with 10 mL of ice-cold 50 mM Tris buffer pH 7.0 containing 1 mg of phenylmethysulfonyl fluoride, and made into a fine paste with the help of a hand held electric blender (Tissue-Tearor™, Bio-Specs Products Inc., Bartlesville, OK, USA). The suspension was immediately centrifuged at 3000 rpm and 4 °C for 10 min, and the clear supernatant was kept on ice until needed for a biochemical assay.

2.5 Assay of Plasma Glucose (GLC)

The plasma glucose was measured using a commercial assay kit (Procedure No. 510, Sigma-Aldrich, St. Louis, MO, USA) based on the glucose oxidase-peroxidase colorimetric method of Raabo and Terkildsen (1960). The results were expressed in mg/dL.

2.6 Assay of Plasma Insulin (INS)

The concentration of circulating INS was measured with a commercial immunoassay kit (ELISA kit, item No. IS130D, Calbiotech Inc., Spring Valley, CA, USA) and an ELISA plate reader set at 450 nm. The results were expressed in μIU/mL.

2.7 Blood Glycated Hemoglobin (HbA_{1c})

This parameter of long term glycemic control was measured with the help of a commercial assay kit (Glycohemoglobin Test, reference No. 0350–060, Stanbio Laboratory, Boerne, TX, USA) based on an ion-exchange resin procedure and measuring both HbA_{1c} and nonglycated (HbA_1) hemoglobin. The results were expressed as a percentage of the total hemoglobin concentration.

2.8 Plasma Transforming Growth Factor-β1 (TGF-β1)

This cytokine was measured in plasma samples by a commercially available ELISA kit (Novex™ Multispecies TGF-β1, Cat. No. KAC 1688, Thermo Fisher Scientific Inc., Waltham, MA, USA). The concentration of TGF-β1 in the sample was derived from an appropriate calibration curve, and the result was expressed in pg/mL.

2.9 Plasma Creatinine (CRN)

This parameter of renal function was measured with the help of a commercial endpoint/enzymatic-colorimetric assay kit (Creatinine LiquiColor® Test, reference No. 0430–500, Stanbio Laboratory, Boerne, TX, USA) based on the Jaffe reaction according to Toora and Rajagopal (2002). The results were expressed as mg/dL.

2.10 Plasma Urea Nitrogen (UN)

This parameter of renal function was measured with a commercial assay kit (Liqui-UV® Test, reference No. 2020–430, Stanbio Laboratory, Boerne, TX, USA) using a published method based on the urease-Berthelot reagent (Tobacco et al. 1979). The results were expressed as mg/dL.

2.11 Plasma and Urine Sodium (Na^+)

The concentration of this electrolyte was measured using a commercial assay kit (Sodium Test, reference No. 0140–050, Stanbio Laboratory, Boerne, TX, USA) in which the fading of the yellow color of uranyl acetate, measured with a spectrophotometer at 420 nm, is proportional to the Na^+ content of the sample. The results were expressed as mM/L.

2.12 Plasma and Urine Potassium (K^+)

The concentration of this electrolyte was measured using a commercial turbidimetric assay kit (Potassium Test, reference No. 0160–050, Stanbio Laboratory, Boerne, TX, USA) in which K^+ is reacted with an alkaline sodium tetraphenylboron reagent, and the absorbance of the solution is read on a spectrophotometer at 580 nm. The results were expressed as mM/L.

2.13 Urine Total Protein (TP)

This biochemical parameter was measured using a commercial assay kit (Total Protein Liquicolor® Test, reference No. 0250–500, Stanbio Laboratory, Boerne, TX, USA) based on the biuret colorimetric reaction as described by Weichselbaum (1946). The results were expressed as g/dL.

2.14 Kidney Malondialdehyde (MDA)

The concentration of MDA was measured as thiobarbituric acid reactive substances (TBARS) after reaction of the test sample with a reagent containing thiobarbituric acid in an acid medium and the experimental conditions described by Buege and Aust (1978). The concentration of MDA was derived from a calibration curve of MDA generated from serial dilutions of a solution of 1,1,3,3-tetraethoxypropane (5–100 nM), and which were treated in identical manner as the test sample. The results were expressed as nM/g of tissue.

2.15 Kidney Reduced Glutathione (GSH) and Glutathione Disulfide (GSSG)

The levels of these two intracellular components were measured by the fluorometric method of Hissin and Hilf (1976), which is based on the reaction of GSH with *ortho*-phthalaldehyde at pH 8.0 and of GSSG at pH 12.0. Prior to the assay of the GSSG, any preformed GSH was removed by complexation with N-ethylmaleimide as described by Güntherberg and Rost (1966). The concentrations of both GSH and GSSG were expressed as μM/g of tissue.

2.16 Kidney Catalase (CAT) Activity

The activity of this enzyme was measured using the spectrophotometric method of Aebi (1984), which is based on the catalase-mediated degradation of hydrogen peroxide to water and oxygen. The results are reported as U/min/g of kidney sample.

2.17 Kidney Glutathione Peroxidase (GPx)

The activity of this GSH-dependent enzyme was determined by the spectrophotometric method of Flohé and Günzler (1984) and its activity in the sample was expressed as μM of NADPH converted to $NADP^+$/min/g of kidney sample.

2.18 Kidney Superoxide Dismutase (SOD) Activity

This enzyme was assayed using the spectrophotometric method of Misra (1985) and its activity in the sample was expressed as U/min/g of kidney sample.

2.19 Statistical Analyses

The experimental results are reported as mean ± SEM for groups of 6 rats each. Differences between groups were analyzed for statistical significance using unpaired Student's t-test and a commercial computer-based statistics program (GraphPad Prism® Version 4.0 from GaphPad Software, Inc., San Diego, CA, and SigmaStat® from Systat Sofware, Inc., San Jose, CA, USA), followed by one-way analysis of variance (ANOVA) and Tukey's post hoc test. Intergroup differences were considered to be statistically significant when $p \leq 0.05$).

3 Results

3.1 Plasma GLC

At the end of 8 weeks, diabetic rats demonstrated a very high (+313%, $p < 0.001$) plasma GLC level compared to normal rats (Table 1). Less marked increases were seen in diabetic rats receiving CAP (+242%), TAU (+222%) or better MET (+90%) at the end of a 6 weeks treatment (all at $p < 0.001$ vs. control). A combined treatment with CAP-MET lowered the diabetic plasma GLC to about the same extent as MET alone (+96%). Adding TAU to a treatment with CAP (+199%) and MET (+84%) led to a moderate and negligible increase, respectively, in hypoglycemic action relative to CAP and MET alone. However, a still greater effect was attained when the three compounds were made available together (+70%) but not with CAP-MET (+96%) (all comparisons vs. control were significant at $p < 0.001$, Table 1). In contrast, none of the oral treatment agents altered the control plasma GLC level to a significant extent.

Table 1 The effects of CAP, MET and TAU, singly and in combination, on the plasma GLC and INS and blood HbA_{1c} levels of diabetic rats[a,b]

Group	Plasma GLC, mg/dL	Plasma INS, μIU/mL	HbA_{1c}, %
Control	$103.59 \pm 5.06^{+++}$	$44.08 \pm 2.46^{+++}$	$6.89 \pm 0.11^{+++}$
DM	$428.08 \pm 21.77^{***}$	$10.58 \pm 1.33^{***}$	$21.14 \pm 1.19^{***}$
DM-CAP	$354.23 \pm 11.89^{***,+}$	$27.61 \pm 3.22^{***,+++}$	$4.20 \pm 1.81^{***,++}$
DM-MET	$196.41 \pm 5.00^{***,+++}$	$32.50 \pm 2.365^{**,+++}$	$7.77 \pm 0.41^{+++}$
DM-TAU	$333.27 \pm 6.60^{***,+}$	$20.94 \pm 1.83^{***,+++}$	$10.96 \pm 0.96^{***,+++}$
DM-CAP-MET	$202.62 \pm 3.51^{***,+++}$	$33.86 \pm 1.72^{**,+++}$	$8.73 \pm 0.92^{*,+++}$
DM-CAP-TAU	$310.03 \pm 5.05^{***,+}$	$38.00 \pm 1.21^{+++}$	$12.31 \pm 0.97^{***,+++}$
DM-MET-TAU	$190.43 \pm 2.22^{***,+++}$	$36.02 \pm 1.87^{*,+++}$	$7.65 \pm 1.04^{+++}$
DM-CAP-MET-TAU	$176.54 \pm 19.17^{***,+++}$	$39.09 \pm 1.93^{+++}$	$8.43 \pm 0.23^{*,+++}$

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

[b]Statistical comparisons were vs. Control rats at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; and vs. DM rats at $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$

3.2 Plasma INS

Diabetes caused the plasma INS to markedly decrease to a value that, by the end of week 8, was markedly below the control value (−76%, $p < 0.001$) (Table 1). In contrast, a treatment with CAP, MET, TAU and their binary and ternary combinations resulted in higher plasma INS levels, with MET (−26%, $p < 0.05$) appearing much more effective than either CAP (−37%, $p < 0.01$) or TAU (−52%, $p < 0.001$). When used as binary combinations, adding TAU to a treatment with CAP or MET enhanced the effects of these drugs further, with CAP-TAU (−14%) providing an insignificantly greater effect than MET-TAU (−18%, $p < 0.05$). In contrast, while the combination CAP-MET (−23%, $p < 0.05$) was equipotent with MET alone, a treatment with MET-CAP-TAU led to a plasma INS value (−11%) that was insignificantly different from the control value (Table 1). None of the treatment agents were found to alter the basal plasma INS to a significant extent (≤3% increase).

3.3 Blood HbA_{1c}

Measurement of the HbA_{1c} level to determine the average blood glucose over a prolonged period of time is a useful way to assess the effectiveness of therapy of diabetes, with values above being 7% considered to represent poor glucose control for most human patients (American Diabetes Association 2014) and for rats of the same strain and age as those used here (Ahmadvand 2012). In the present study untreated diabetic rats demonstrated a blood HbA_{1c} that was more than 200% greater than the control value ($p < 0.001$, Table 1). Treating the diabetic rats with MET resulted in a higher attenuation of the diabetic plasma glucose (only +13%) than one with TAU (+59%, $p < 0.001$) or CAP (+106%, $p < 0.001$). Use of binary combinations led to a marked improvement in the suppression of HbA_{1c} formation, with MET-TAU (only +11%) appearing as the most effective, CAP-MET providing an intermediate potency (+27%, $p < 0.05$) and CAP-TAU exerting the lowest effect (+79%, $p < 0.001$). On the other hand, providing the diabetic rats with CAP-MET-TAU also resulted in a significant decrease of the blood HbA_{1c} level (+22%, $p < 0.05$) although not to the same extent as a treatment with MET-TAU (Table 1). When given to normal rats, CAP, MET and TAU did not alter the basal blood HbA_{1c} level.

3.4 Plasma TGF-β1

This indicator of diabetic microvascular and macrovascular complications was significantly increased in the plasma of diabetic rats (>12-fold, $p < 0.001$) relative to the control value (Table 2). A treatment of diabetic rats with CAP, MET or TAU led

Table 2 The effects of CAP, MET and TAU, singly and in combination, on the plasma TGF-β1 levels of diabetic rats[a,b]

Group	Plasma TGF-β1, pg/mL
Control	$43.64 \pm 11.22^{+++}$
DM	$543.21 \pm 47.71^{***}$
DM-CAP	$143.93 \pm 33.71^{***, +++}$
DM-MET	$167.46 \pm 15.68^{***,+++}$
DM-TAU	$168.18 \pm 21.99^{***, +++}$
DM-CAP-MET	$104.79 \pm 10.06^{***,+++}$
DM-CAP-TAU	$132.41 \pm 10.07^{***,+++}$
DM-MET-TAU	$141.75 \pm 15.85^{***,+++}$
DM-CAP-MET-TAU	$72.64 \pm 6.90^{***,+++}$

[a]Values are reported as the mean ± SEM for n = 6
[b]Statistical comparisons were vs. Control rats at $^{***}p < 0.001$; and vs. DM rats at $^{+++}p < 0.001$

to attenuation of this rise, with CAP (3.3-fold, $p < 0.001$) providing a greater attenuation than either MET or TAU (~3.85-fold increase with both, $p < 0.001$). Adding TAU to a treatment with either CAP or MET led to different effects, being negligible with CAP-TAU (threefold increase, $p < 0.001$) and to an increase with MET-TAU (~3.25-fold, $p < 0.001$) relative to a treatment without TAU. However, a greater decrease in plasma TGF-β1 levels was attained with CAP-MET (2.4-fold increase, $p < 0.001$) and especially with CAP-MET-TAU (only 1.7-fold increase, $p < 0.001$) (Table 2). When given to normal rats, these compounds were found not to alter the plasma TGF-β1 values of normal rats to a significant extent.

3.5 Plasma CRN and UN

At the end of 8 weeks, diabetic rats showed a much higher plasma CRN (+332%, $p < 0.001$) level than control rats (Table 3). A treatment of these rats with CAP (+71%), MET (+139%) or TAU (+100%) led to a significant reduction of the diabetic plasma CRN level (all at $p < 0.001$ vs. diabetes). Furthermore, a daily treatment with the combinations CAP-TAU (+77%) and MET-TAU (+148%) did not enhance the actions of either CAP or TAU further; and one with CAP-MET (+119%) was marginally better than one with MET alone; but providing the diabetic rats with the three compounds together led to a drastic gain in potency (only +39%, $p < 0.01$) over monotherapy or bitherapy (Table 3).

In parallel with an increase in plasma CRN, diabetes also a drastic (+158%, $p < 0.001$ vs. control) increase in the plasma UN at the end of a 8 weeks period (Table 3). Treating the diabetic rats with TAU (+68%) or better with CAP (+41%), was more effective than with MET (+102%, $p < 0.001$) in lowering this elevation; but a combined treatment with TAU enhanced the effect of both MET (only +23%, $p < 0.05$) and CAP (+34%, $p < 0.01$) than either treatment alone. While a combined treatment with CAP-MET (+39%, $p < 0.01$) provided an attenuating effect approximating that of CAP-TAU (+34%, $p < 0.01$), one with CAP-MET-TAU was

Table 3 The effects of CAP, MET and TAU, singly and in combination, on the plasma CRN, UN and TP levels of diabetic rats[a,b]

Group	Plasma CRN, mg/dL	Plasma UN, mg/dL	Plasma TP, g/dL
Control	$0.31 \pm 0.03^{+++}$	$11.31 \pm 0.62^{+++}$	$9.25 \pm 0.26^{+++}$
DM	$1.34 \pm 0.02^{***}$	$29.14 \pm 1.16^{***}$	$4.37 \pm 0.55^{***}$
DM-CAP	$0.53 \pm 0.06^{***,+++}$	$15.90 \pm 1.14^{***,+++}$	$6.50 \pm 0.39^{**,+++}$
DM-MET	$0.74 \pm 0.09^{***,+++}$	$22.80 \pm 0.90^{***,++}$	$5.96 \pm 0.43^{**,++}$
DM-TAU	$0.62 \pm 0.06^{***,+++}$	$18.74 \pm 1.27^{***,++}$	$7.03 \pm 0.69^{**,+++}$
DM-CAP-MET	$0.68 \pm 0.01^{***,+++}$	$15.72 \pm 1.22^{**,+++}$	$6.00 \pm 0.37^{**,++}$
DM-CAP-TAU	$0.55 \pm 0.01^{***,+++}$	$15.10 \pm 0.23^{**,+++}$	$7.11 \pm 0.31^{**,+++}$
DM-MET-TAU	$0.77 \pm 0.09^{***,+++}$	$13.89 \pm 1.53^{**,+++}$	$6.46 \pm 0.42^{**,+++}$
DM-CAP-MET-TAU	$0.43 \pm 0.08^{**,+++}$	$12.42 \pm 0.93^{+++}$	$7.11 \pm 0.11^{***,+++}$

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

[b]Statistical comparisons were vs. Control (normal) rats at $^{**}p < 0.01$ and $^{***}p < 0.001$; and vs. diabetic (DM) rats at $^{++}p < 0.01$ and $^{+++}p < 0.001$

still more effective (+10% increase) (Table 3). None of the treatment compound were found to alter the basal plasma CRN and UN levels to a significant extent.

3.6 Plasma TP

In comparison to control rats, diabetic ones showed a large decrease in the plasma TP level (−53%, $p < 0.001$) at the end of 8 weeks (Table 3). A treatment of these rats with TAU (−22%, $p < 0.05$) reduced the loss of the plasma TP to a greater extent than one with either CAP (−30%, $p < 0.01$) or MET (−36%, $p < 0.01$). A combined treatment with CAP-TAU (−23%, $p < 0.05$) was as effective as TAU alone, but slightly better than either MET-TAU (−30%, $p < 0.01$) or CAP-MET (−35%, $p < 0.01$); and providing the diabetic rats with MET-CAP-TAU led to an equivalent effect as one with CAP-TAU (Table 3). None of the treatment agents was found not to reduce the plasma TP level of control rats by more than 10%.

3.7 Plasma and Urine Na^+

At the end of 8 weeks diabetic rats exhibited a higher level of plasma Na^+ than control rats of equivalent weight (+35%, $p < 0.01$) (Table 4). A daily treatment of the diabetic rats with CAP, MET or TAU from day 15 onwards led to much lower circulating Na^+ levels (+2% with CAP, +11% with MET and TAU), an effect that was enhanced further when TAU was co-administered with either CAP or TAU (no difference from the

Table 4 The effects of CAP, MET and TAU, singly and in combination, on the plasma and urine Na^+ levels of diabetic rats[a,b]

Group	Plasma Na^+, mM/L	Urine Na^+, mM/L	Plasma/urine Na^+ ratio
Control	$144.45 \pm 6.42^{+++}$	$19.23 \pm 3.40^{+++}$	1.21^{+++}
DM	$194.82 \pm 1.06^{**}$	$80.23 \pm 2.93^{**}$	2.43^{***}
DM-CAP	$147.60 \pm 2.76^{++}$	$99.27 \pm 1.71^{*,++}$	$1.49^{*,++}$
DM-MET	$160.36 \pm 2.84^{+}$	$101.88 \pm 3.42^{*,++}$	$1.57^{*,+}$
DM-TAU	$159.81 \pm 2.46^{+}$	$94.70 \pm 2.33^{**,+}$	$1.69^{**,+}$
DM-CAP-MET	$165.18 \pm 5.62^{*,+}$	$96.30 \pm 3.16^{*,++}$	$1.72^{**,+}$
DM-CAP-TAU	$144.79 \pm 4.62^{++}$	$103.21 \pm 7.38^{*,++}$	$1.40^{*,++}$
DM-MET-TAU	$147.40 \pm 3.41^{++}$	$98.30 \pm 4.23^{*,++}$	$1.50^{*,++}$
DM-CAP-MET-TAU	$151.38 \pm 3.71^{++}$	$10.71 \pm 2.81^{+++}$	1.37^{++}

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

[b]Statistical comparisons were vs. Control rats at $^{*}p < 0.05$, and $^{**}p < 0.01$; and vs. DM rats at $^{+}p < 0.05$ and $^{++}p < 0.01$

control value) or as a combination with both CAP and TAU (+5%); and a treatment with CAP-MET (+2%) was about equipotent to one with CAP-MET-TAU (+5%) (Table 4).

As expected, the increase in plasma Na^+ caused by diabetes was accompanied by a significant fall in the urinary excretion of Na^+ (−39%, $p < 0.01$ vs. control) (Table 4). In contrast, treating the diabetic rats with CAP, MET or TAU led to an enhanced renal excretion of Na^+, with the effect being quantitatively rather similar (−14%, −15% and −12%, respectively); and the effectiveness of a binary treatment with CAP-TAU (−13%), MET-TAU (−18%, $p < 0.05$) or CAP-MET (−19%, $p < 0.05$) was about equal to that attained using monotherapy. In contrast, providing the diabetic rats with CAP-MET-TAU reduced the urinary loss of Na^+ to a value comparable to the value for control rats (only −7%) (Table 4). All the treatment agents and their various combinations were able to reduce the increase in plasma Na^+/urine Na^+ ratio seen in diabetic rats (+100%, $p < 0.001$ vs. control) to a significant extent, with CAP, CAP-TAU and MET-TAU providing a stronger lowering effect (only16–24% increases, $p < 0.05$) than MET, TAU or CAP-MET (40–43% increases, $p < 0.01$) on the diabetic ratio. On the other hand, CAP-MET-TAU was able to lower the ratio to a value not significantly different from the control value (+13%) (Table 4). None of the treatment compounds altered the urinary excretion of Na^+ to a significant extent.

3.8 Plasma and Urine K^+

By analogy to results for the plasma and urine Na^+ levels, those for K^+ were also increased and decreased, respectively, when compared to the corresponding control values (Table 5). In the case of the plasma K^+, diabetic rats retained a

Table 5 The effects of CAP, MET and TAU, singly and in combination, on the plasma and urine K^+ levels of diabetic rats[a,b]

Group	Plasma K^+, mM/L	Urine K^+, mM/L	Plasma/urine K^+ ratio
Control	$10.24 \pm 0.41^{++}$	$6.95 \pm 34.61^{++}$	0.18^{+++}
DM	$16.02 \pm 0.45^{***}$	$34.61 \pm 1.14^{***}$	0.46^{***}
DM-CAP	$11.40 \pm 0.39^{*,++}$	$45.74 \pm 1.25^{*,++}$	$0.25^{**,+++}$
DM-MET	$11.90 \pm 0.67^{**,+}$	$45.23 \pm 3.47^{*,++}$	$0.26^{***,+++}$
DM-TAU	$11.01 \pm 6.60^{++}$	$49.26 \pm 3.69^{+++}$	$0.22^{*,+++}$
DM-CAP-MET	$11.23 \pm 0.21^{++}$	$46.31 \pm 2.34^{*,++}$	$0.24^{**,+++}$
DM-CAP-TAU	$11.06 \pm 0.91^{++}$	$48.31 \pm 4.12^{*,++}$	$0.23^{*,+++}$
DM-MET-TAU	$11.07 \pm 0.42^{++}$	$45.31 \pm 3.68^{**,++}$	$0.24^{**,+++}$
DM-CAP-MET-TAU	$10.50 \pm 0.71^{++}$	$50.21 \pm 3.04^{+++}$	$0.21^{*,+++}$

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

[b]Statistical comparisons were vs. Control rats at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$; and vs. DM rats at $^{++}p < 0.01$ and $^{+++}p < 0.001$

greater concentration of K^+ (56%, $p < 0.001$) than normal rats. However, a daily treatment of these diabetic rats with CAP, MET or TAU lowered the plasma K^+ to a value that was comparable to the control value (+8%, +16% and +8%, respectively). On the other hand, the administration of TAU together with CAP or MET led to results identical to those seen with TAU alone (i.e., +8%), a situation that was also attained with CAP-MET (+10%). Providing the diabetic rats with the three compounds resulted in virtually normal (+2.5%) plasma K^+ values (Table 5). None of the treatment agents altered the baseline plasma K^+ values.

At variant with the increase in K^+ levels seen in the plasma of diabetic rats, the corresponding urine levels were significantly below the control value (−46%, $p < 0.001$) (Table 5). Treating the diabetic rats with CAP, MET or TAU reduced the urine K^+ by more than one-half of the diabetic value (to ~20% with both CAP and MET, $p < 0.05$; to −14% with TAU), an effect that was also seen when CAP and MET were each administered alongside TAU (−15% with CAP-TAU, −20% with MET-TAU, $p < 0.05$) or when given together (−20%, $p < 0.05$). However, administering the three compounds together led to a urine K^+ value that was not significantly different from that of control rats (−12%) (Table 5). As verified for the plasma Na^+/urine Na^+ ratio, that for plasma K^+/urine K^+ was also significantly increased in diabetic rats (+156%, $p < 0.001$ vs. control), with TAU (only +22%, $p < 0.05$) and CAP-TAU (+28%, $p < 0.5\%$) lowering the diabetic increase to a greater extent than the other treatments (+33–44%, $p < 0.01$). Providing the diabetic rats with CAP-MET-TAU led to a further, although small, reduction of the diabetic plasma K^+/urine K^+ ratio (+17%, $p < 0.05$) (Table 5) The administration of all of the test compounds to normal rats did not have a significant effect on the urine K^+ level.

Table 6 The effects of CAP, MET and TAU, alone and in combination, on the kidney MDA levels of diabetic rats[a,b]

Group	MDA, mM/g
Control	$3.94 \pm 0.07^{++}$
DM	$5.61 \pm 0.08^{***}$
DM-CAP	$4.25 \pm 0.07^{++,}$
DM-MET	$3.97 \pm 0.04^{++}$
DM-TAU	$3.99 \pm 0.07^{++}$
DM-CAP-MET	$3.77 \pm 0.05^{+++}$
DM-CAP-TAU	$3.66 \pm 0.06^{++}$
DM-MET-TAU	$3.81 \pm 0.04^{++}$
DM-CAP-MET-TAU	$3.72 \pm 0.02^{++}$

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

[b]Statistical comparisons were vs. Control rats at $^{***}p < 0.001$; and vs. DM rats at $^{++}p < 0.01$

3.9 Kidney MDA

In comparison to normal rats, untreated diabetic ones showed a higher renal level of MDA (+41%, $p < 0.01$) (Table 6). Without exceptions, a treatment of these rats with CAP, MET or TAU brought the MDA levels to values that were similar to the baseline values (≤8%), an effect that was also achieved when CAP (−7%) or MET (−3%) were paired with TAU or with each other (−4%) or when the three compounds were given together (−6%) (Table 6). None of the treatment compounds showed an effect on the baseline kidney MDA levels.

3.10 Kidney GSH, GSSG and GSH/GSSG Ratio

Diabetic rats exhibited a moderate decrease in renal GSH (−34%, $p < 0.01$) and a very high drop in the renal GSSG level (+127%, $p < 0.001$) by the end of week 8 relative to corresponding control values (Table 7). These values were attenuated by CAP (−26%), MET (−20%) and TAU (−27%) but not to a significant extent unless CAP (−21%) or MET (−9%) were paired with TAU. or with each other (−7%). By contrast, a combined treatment with the three compounds reversed the deficit (+13%, $p < 0.01$ vs. diabetes) (Table 7). When given to normal rats, none of the test compounds were found to affect the basal GSH levels significantly.

In the case of the GSSG levels, treating the diabetic rats with either CAP (+42%), MET (+83%) or TAU (+13%) resulted in a significant ($p \leq 0.01$) reduction in the formation of GSSG caused by diabetes (Table 7); but treating the diabetic rats with either CAP-TAU (+56%) or MET-TAU (+54%) led to a lowering of the protective

Table 7 The effects of CAP, MET and TAU, alone and in combination, on the kidney GSH and GSSG levels and GSH/GSSG ratio of diabetic rats[a,b]

Group	GSH, nM/g	GSSG, nM/g	GSH/GSSG ratio
Control	$95.98 \pm 1.76^{++}$	$4.80 \pm 0.23^{+++}$	$19.99 \pm 0.08^{+++}$
DM	$63.34 \pm 0.58^{***}$	$10.88 \pm 045^{***}$	$5.82 \pm 0.03^{***}$
DM-CAP	$70.79 \pm 1.58^{**,+}$	$6.83 \pm 0.72^{***,++}$	$10.37 \pm 0.04^{***,+++}$
DM-MET	$76.33 \pm 1.55^{**,++}$	$8.81 \pm 0.25^{***,+}$	$8.66 \pm 0.04^{***,+++}$
DM-TAU	$69.59 \pm 1.08^{**,+}$	$5.43 \pm 0.21^{*,+++}$	$12.81 \pm 0.04^{**,+++}$
DM-CAP-MET	$89.17 \pm 1.92^{++}$	$8.73 \pm 0.92^{*,+++}$	$12.28 \pm 0.04^{**,+++}$
DM-CAP-TAU	$75.99 \pm 0.83^{*,+}$	$7.50 \pm 0.68^{***,++}$	$10.13 \pm 0.06^{***,+++}$
DM-MET-TAU	$87.73 \pm 2.20^{++}$	$7.39 \pm 0.61^{***,++}$	$11.87 \pm 0.06^{***,+++}$
DM-CAP-MET-TAU	$108.55 \pm 1.93^{+++}$	$5.63 \pm 0.18^{+++}$	$19.28 \pm 0.06^{+++}$

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

[b]Statistical comparisons were vs. Control rats at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$; and vs. DM rats at $^{+}p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$

effect achieved with either compound alone, which was also the case when CAP and MET were given concurrently (−82%, $p < 0.001$ vs. control). In contrast, treating the diabetic rats with MET-CAP-TAU resulted in a renal GSSG value that was approximated that seen with TAU alone (+17% (Table 7). None of the treatment agents was found to affect the renal GSSG levels of normal rats to a significant extent.

Based on the renal values for GSH and GSSG, it was determined that in diabetic rats the intracellular redox state, expressed as the GSH/GSSG ratio, was drastically reduced in the kidney of diabetic rats (−71%, $p < 0.001$) relative to the control value (Table 7). This ratio was found to rise following a treatment with either MET (−57%, $p < 0.001$), CAP (−48%, $p < 0.001$) and particularly with TAU (−35%, $p < 0.01$).

Treating the diabetic rats with MET-TAU (−41%) and CAP-MET (−39%), but not with CAP-TAU (−49%), led to an improvement of the effect attained with monotherapy ($p < 0.01$ vs. control). This gain was further enhanced when the diabetic rats were fed MET-CAP-TAU, at which point the GSH/GSSG ratio became comparable to the control value (−4% decrease) (Table 7). None of the treatment agents affected the basal renal GSH/GSSG significantly.

3.11 Kidney CAT

The renal activity of CAT was grossly reduced in diabetic rats (−71%, $p < 0.001$) relative to control rats (Table 8). A 6 weeks treatment with CAT (−41%, $p < 001$), MET (−50%, $p < 0.001$) and especially TAU (−29%, $p < 0.05$) led to a significant attenuation of this effect. The addition of TAU to a treatment with CAP (−10%) or MET (−18%, $p < 0.05$) enhanced the attenuating effect of these compounds further. This effect was even greater when the treatment was with CAP-MET (−7%) or

Table 8 The effects of CAP, MET and TAU, alone and as combinations, on the kidney CAT, GPx and SOD activities of diabetic rats[a,b]

Group	CAT, U/min/g tissue	GPx U/min/g tissue	SOD U/min/g tissue
Control	$0.84 \pm 0.02^{+++}$	$0.94 \pm 0.06^{+++}$	$1.50 \pm 0.22^{+++}$
DM	$0.24 \pm 0.01^{***}$	$0.28 \pm 0.02^{***}$	$0.23 \pm 0.06^{***}$
DM-CAP	$0.52 \pm 11.89^{**,+++}$	$0.69 \pm 0.03^{**,+++}$	$0.88 \pm 0.05^{***,+++}$
DM-MET	$0.42 \pm 0.03^{***,+++}$	$0.75 \pm 0.06^{**,+++}$	$1.09 \pm 0.06^{**,+++}$
DM-TAU	$0.60 \pm 0.04^{**,+++}$	$0.83 \pm 0.03^{+++}$	$0.97 \pm 0.01^{**,+++}$
DM-CAP-MET	$0.78 \pm 0.05^{+++}$	$0.84 \pm 0.01^{**,+++}$	$1.02 \pm 0.08^{**,+++}$
DM-CAP-TAU	$0.76 \pm 0.05^{+++}$	$0.77 \pm 0.02^{*,+++}$	$1.04 \pm 0.05^{**,+++}$
DM-MET-TAU	$0.69 \pm 0.01^{*,+++}$	$0.84 \pm 0.01^{**,+++}$	$1.00 \pm 0.04^{**,+++}$
DM-CAP-MET-TAU	$0.99 \pm 0.06^{*,+++}$	$0.90 \pm 0.03^{+++}$	$1.38 \pm 0.03^{+++}$

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

[b]Statistical comparisons were vs. Control (normal) rats at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$; and vs. Diabetic (DM) rats at $^{+++}p < 0.001$

better when CAP-MET and TAU were made available together, in which case the effect of diabetes on the renal CAT activity was totally reversed (+18%, $p < 005$) (Table 8). On the other hand, none of the treatment compounds was found to affect the basal renal CAT activity to a significant extent.

3.12 Kidney GPx

In comparison to normal rats, diabetic ones showed a profound decrease (−70%, $p < 0.001$) in renal GPx activity (Table 8). A daily treatment of the diabetic rats with MET (−20%, $p < 0.05$), CAP (−27%, $p < 0.05$) and especially TAU (−12%) resulted in a strong attenuation of the diabetic effect. The protective effect was enhanced by adding TAU to a treatment with either CAP (−18%, $p < 0.05$) or MET (−11%) or by a combined treatment with CAP-MET (−11%). An additional gain in potency was achieved by treating the diabetic rats with these three compounds, in which case an almost complete reversal of the diabetic effect was achieved (only 4% decrease) (Table 8). None of the treatment agents had a significant effect on the basal kidney GPx activity.

3.13 Kidney SOD

By analogy to the effect of diabetes on other antioxidant enzymes, after 8 weeks the renal activity of SOD in diabetic rats was also significantly reduced (−85%, $p < 0.001$) compared to the control group (Table 8). A 5 weeks treatment of these rats with CAP (−44%, $p < 0.001$), MET (−27%, $p < 0.05$) or TAU (−35%, $p < 0.01$)

reduced this effect significantly. A combined treatment with CAP-TAU (−31%, $p < 0.01$), CAP-MET (−32%) or MET-TAU (−33%, $p < 0.01$) led to an effect approximating that seen with TAU alone; and treating the diabetic rats with these three compounds led to an almost complete restoration of the basal SOD activity (−8%) (Table 8). The administration of the treatment compounds to normal rats resulted in different degrees of elevation, being insignificant with MET +11%) and TAU (+13%) and significant with CAP (+20%, $p < 0.05$).

4 Discussion

The present study has examined the effects of a 6 weeks treatment with either CAP, MET or TAU and their binary and ternary combinations on biochemical indices of GLC metabolism and kidney function and oxidative stress associated with STZ-induced DM. The dose of STZ used in the study was one found to cause a persistent glucose level that exceeded 350 mg/dL soon after its administration (Gajdošik et al. 1999).

Untreated diabetic rats showed a plasma GLC level that was more than fourfold higher than that of normal rats. Although treatment with a single agent was able to reduce the diabetic GLC levels, MET was at least 2.5-times more effective than either TAU or CAP in that order, an effect that was enhanced when CAP and MET were given along with TAU, more with CAP than with MET. While the hypoglycemic action of MET has been related to an increase in peripheral INS sensitivity and, hence, to GLC utilization, to inhibition of hepatic gluconeogenesis, and to a reduction of gastrointestinal GLC absorption, but without stimulating INS secretion (Davidson and Peters 1997; Viollet et al. 2012), less is known on how CAP negatively influences the circulating GLC levels. One possibility may be through an increase in peripheral INS sensitivity without an effect on INS secretion (Pollare et al. 1989; Rett et al. 1986); and another is through an ability to promote the uptake GLC by the skeletal muscle for eventual metabolic disposal (Kodama et al. 1990; Rett et al. 1986). Regardless of the exact mechanism underlying the hypoglycemic action of CAP, this issue is still unsettled since another study found CAP to neither influence GLC tolerance nor INS sensitivity in hypertensive patients with type 2 DM (Yin et al. 1994). TAU is amino acid reported to exert a hypoglycemic effect by increasing INS availability, by promoting hepatic GLC accumulation as glycogen (Gavrovskaya et al. 2008), and by lowering the rate of renal gluconeogenesis (Koh et al. 2014) in animal models of diabetes. In spite of the numerous studies describing the beneficial effects of TAU on the blood GLC and INS secretion (de Oliveira et al. 2011), resistance (Kim et al. 2012) or sensitivity (Brøns et al. 2004; Nakaya et al. 2000), there are also reports indicating a lack thereof (Brøns et al. 2004; Goodman and Shihabi 1990). The present study determined that although TAU lowered the plasma GLC of diabetic rats to a greater extent than CAP, this effect was less than one-half of that shown by MET. Furthermore, when co-administered with CAP or MET this amino acid was found to enhance the effect of CAP, but not of MET, mildly. In any case, while both treatment combinations were slightly more

effective in lowering the plasma GLC of diabetic rats than MET-CAP, a combined treatment with all three compounds was the only one to reduce the diabetic plasma GLC to within control values.

While diabetes reduced the plasma INS to about one-fourth the normal level, a treatment with either CAP, MET or TAU was found to counteract this effect to a significant but different extents. Thus, MET was found to produce the highest increase of the plasma INS in diabetic animals, followed by CAP, with TAU appearing as the least potent. The effect of MET on INS secretion may reflect an ability to reestablish the INS secretory function of the pancreas that became impaired by a chronic exposure to the high levels of free fatty acids and GLC typically seen in diabetes (Patané et al. 2000). This effect, has been described as a desensitization of pancreatic islets as a result of a prolonged exposure to high concentrations of GLC (Lupi et al. 1999). The present finding for CAP contrasts markedly with those reported by Jasik et al. (1996) and indicating that the hypoglycemic action of this ACE inhibitor is exerted through an improvement of INS sensitivity rather than of any influence on INS secretion by pancreatic β-cells. In contrast, an elevating effect on the plasma INS by an ACE inhibitor has been previously reported by Roysommuti et al. (2013). In parallel with CAP, the effect of TAU on INS secretion is also surrounded by controversy, with some studies describing an increasing effect (L'Amoreaux et al. 2010; Ribeiro et al. 2009), some an inhibitory effect (Wang et al. 2008), and still some reporting no effect on either secretion or sensitivity to the hormone (Brøns et al. 2004). Regardless of the exact mechanism by which TAU lowers the circulating GLC, the present study found that even though TAU was able to raise the plasma INS it was, however, much weaker than either MET or CAP. However, when used alongside MET or CAP it was able to enhance the actions of these compounds, more so with CAP than with MET. Although a treatment with CAP-MET was not as potent as one with CAP-TAU or MET-TAU, when available along with TAU, the ternary combination raised the plasma INS to a value not significantly different from the normal value.

The measurement of the HbA_{1c} level is considered to be a useful approach to monitoring long term glycemic control in diabetic patients since it can serve as an indicator of adverse outcome risks. In general, an agreement has been established in human patients between the circulating levels of GLC and those of HbA_{1c} in human patients and with renal function. Indeed, very high levels of blood HbA_{1c} have been closely associated with a rapid decline of renal function and with an increased risk of mortality irrespective of the type of diabetes (Rossing et al. 2004. In the present study, an increase of the plasma GLC in diabetic rats was accompanied by a marked increase of the blood HbA_{1c} relative to the same values for normal rats. Such an increase was lowered by MET to a level insignificantly different from the normal one; but one with either CAP or TAU reduced the diabetic blood HbA_{1c} to a level was much still higher than normal (by twofold and 1.6-fold, respectively). A combined treatment of these compounds with TAU led to an improvement of the effect of CAP (~1.8-fold increase) but not of MET; and using the three compounds together resulted in a HbA_{1c} level that was intermediate to those seen with TAU-CAP and TAU-MET. The present results with MET contrast markedly with those

attained in humans with type 2 DM and in which the success of monotherapy in bringing the HbA_{1c} to a desirable level is much lower than that reported here, especially since drug effectiveness decreases with increasing patient age and its attainment may require a combination therapy (Nosadini 2002; Turner et al. 1999).

TGF-β1 is a fibrogenic cytokine that in addition to being increased in the glomeruli of type 2 diabetic patients (Genc et al. 2010) and in DM-induced diabetic rats it is stimulated by the diabetic process to play a key role in the pathogenesis of DN (Gilbert et al. 1998; Wolf et al. 2005). Specifically, TGF-β1 causes an increase in mesangial matrix deposition, glomerular basement membrane (GBM) thickening, podocyte apoptosis or detachment, and adhesion of the bare GMB to the Bowman's capsule to initiate the process of glomerulosclerosis (Wolf et al. 2005). Conversely, inhibition of the expression of this protein in diabetic *db/db* mice with a neutralizing anti-TGF-β1 antibody has resulted in the prevention of diabetic renal hypertrophy, mesangial matrix expansion and the development of renal insufficiency but without affecting the accompanying albuminuria (Ziyadeh et al. 2000). In the present study, all treatment compounds were able to attenuate the more than 12-fold rise of the plasma TGF-β1 level, caused by diabetes with CAP providing a greater decreasing effect than either MET and TAU. When CAP and MET were given together with TAU, the potency of MET-TAU increased to a somewhat greater extent than that of CAP-TAU (threefold increase) relative to MET and CAP alone. However, the greatest reduction in plasma TGF-β1 was observed in diabetic rats treated with a combination of the three test compounds, in which case the TGF-β1 level was only ~1.7-fold above the control value. Since in subjects with type 2 DM an elevated level of TGF-β1 indicates a tendency for renal damage, the use of TAU along with standard hypoglycemic therapy could be of value in preventing renal diabetic complications.

The decreasing effect of CAP on the plasma TGF-β1 has been previously reported by Sharma et al. (1999) and confirmed *in vitro* by Noh et al. (2005) using cultures of human peritoneal mesothelial cells. Protection by MET against diabetic nephropathy, including fibrosis, has been related to an ability to inhibit the binding of TGF-β1 to its receptor at target sites, thus preventing downstream signal transduction leading to fibrosis (Xiao et al. 2016). In the case of TAU, there is evidence to indicate that the daily consumption of this amino acid by rats with DM-induced diabetes as part of the drinking water (1%, w/v), and started at 4 months after the induction of diabetes, can lower TGF-β1 expression in the renal glomerulus and stabilize the urinary excretion of proteins, effects that have been ascribed, at least in part, to an antioxidant action (Higo et al. 2008). Overall, it has been stated that elevations of the circulating levels of TFG-β1 levels in patients with type 2 DM may indicate a tendency for renal cell damage, and that a lack of changes in the levels of this cytokine after therapy could reflect inadequate therapy duration with an antidiabetic drug such as MET (Yener et al. 2008). Although it is likely that an improvement in the glycemic control of diabetes by MET reduces TGF-β1 levels and, thus, partly contributes to renoprotection (Vinagre et al. 2014), in the case of TAU other factors are probably involved since TAU is intrinsically a much weaker hypoglycemic than MET.

To check the renal function of diabetic rats, the plasma levels of CRN, UN, and TP and the plasma and urine levels of Na^+ and K^+ of diabetic rats were measured at the end of 56 days. CRN and UN are two metabolic waste products that are normally removed from the body by renal excretion. In kidney diseases the renal excretion of these two substances is impaired and, as a result, they tend to accumulate in the circulation. In this work the plasma CRN level of diabetic rats was at least fourfold higher than that of normal rats. Treating the diabetic rats with either CAP, MET or TAU led to reductions of the CRN caused by diabetes, with CAP providing the greatest protection and MET the least. Pairing CAP or MET with TAU resulted in no appreciable gain in potency relative to a treatment with each compound alone, and adding CAP to MET led to an effect intermediate to those of the individual compounds. On the other hand, a combined treatment with MET-CAP-TAU was more potent than any single or binary treatment. While the present findings on the effects of CAP on the serum CRN agree with those described by Akbar et al. (2013) for rats with diabetic nephropathy, they differ from those by Katoh et al. (2000), who found CAP to lower blood pressure and to inhibit urinary albumin excretion but to fail to inhibit renal hypertrophy and elevation of the CRN clearance in STZ-treated rats. In contrast, the present results for TAU confirm those of a previous study that found that daily consumption of this sulfur-containing compound as part of the drinking water reduced the plasma levels of both CRN and UN in diabetic rats (Wang et al. 2008).

In parallel to the effects of CAP, MET and TAU on the diabetic plasma CRN, those on the plasma UN followed a similar trend, with CAP exerting a greater effect than either TAU or MET in that order. While diabetes raised the plasma UN by about 2.6-fold, in the presence of CAP this increase was only 1.4-fold above the control value; and about twofold in the presence of MET. Again TAU showed an intermediate potency (~1.7-fold increase). However, in contrast to the findings for the serum CRN, pairing TAU with either CAP or MET led, in both cases, to a gain in potency in reducing the plasma UN, more with MET-TAU (1.2-fold increase) than with CAP-TAU (1.3-fold increase). As shown for the serum CRN, a treatment of the diabetic rats with CAP-MET led to an potency intermediate to those of the individual compounds, and one with CAP-MET-TAU was able to reduce the plasma UN to a level only 10% above the control value. A study looking at the relationship between an increase in serum GLC with an increase in the circulating levels of CRN and urea in diabetic patients with progressive renal damage determined that it was more consistent with an increase of the latter than of the former compound (Shrestha et al. 2008). Furthermore, over a period of more than 15 years from the time of diagnosis of a case of type 2 diabetes in human patients, further increases in circulating CRN have been associated with the occurrence of nephropathy and macroalbuminuria and with an increased risk of cardiovascular death among those not requiring renal replacement therapy (Adler et al. 2003); although people who lived more than 25 years without any signs of kidney failure may have a decrease risk of developing it (Dabla 2010).

In diabetic rats, the plasma TP level was reduced by about 50% of the control value. This effect was reduced by TAU by more than one-half, and to a lesser,

although still significant extent, by CAP and MET. While a treatment with CAP-MET did not change the potency of MET, one with MET-TAU was marginally better than one with MET alone, and one with CAP-TAU was equipotent with TAU. Since attenuation of the TP loss caused by diabetes by a combined treatment with CAP-MET-TAU was identical to that seen with TAU alone, it is safe to conclude that TAU is the main protective factor. Furthermore, the present results confirm the view that since the progression of diabetic nephropathy is not only dependent on the activity of ACE, renoprotection should be widen to accommodate therapeutic agents with other biological actions (Bernadet-Monrozies et al. 2002). Therapy with CAP has been found to significantly impede progression to clinical proteinuria and to prevent the increase in albumin excretion rate in normotensive patients with INS-dependent diabetes mellitus and persistent microalbuminuria (Viberti et al. 1994); and MET has demonstrated the ability to significantly decrease albuminuria in patients with type 2 DM while ameliorating tubular cell injury (Nasri et al. 2013). The beneficial effects of MET on the kidney of diabetic rats, including albuminuria, could be a consequence of an ability to reduce ROS, to preserve the viability of podocytes or to promote the activation of the enzyme adenosine monophosphate-activated protein kinase, a major regulator of basal and INS-stimulated glucose uptake, lipid and protein synthesis and an inhibitor of complex I of the respiratory chain in the mitochondrion (Kim et al. 2012; Liu et al. 2008). Adding TAU to the drinking water of DM-treated diabetic rats has been reported to reduce total proteinuria and abuminuria by almost 50%, to normalize the renal cortical MDA content and to decrease the formation of advanced glycoxidation products (Trachtman et al. 1995). In common with MET, supplementation of a rat diet with TAU has been reported to suppress the progression of DN through a reduction of renal oxidant injury, LPO, renal accumulation of AGEs (Trachtman et al. 1995) and the development of fibrosis (Koh et al. 2014).

The diabetic rats were found to retain both K^+ and Na^+ in their plasma, with the increase of the former exceeding that of the latter by more than 20%. Treating these rats with CAT, MET and/or TAU was found effective in reducing these accumulations which, in general, amounted to values not exceeding the control plasma K^+ and Na^+ levels by more than 19% and 11%, respectively, with the individual compounds and becoming virtually normal when given as a binary or ternary treatment combination. Relative to the diabetic plasma levels of K^+ and Na^+, those in the urine were significantly lower than corresponding control values, with the extent of the reductions being rather similar for both K^+ and Na^+. As was the case with the diabetic plasma levels of Na^+ and K^+, CAP, MET and/or TAU were also found to effectively counteract the urinary decreases of K^+ and Na^+, which roughly was reduced by at least one-half of those seen in diabetic rats. In general, the potency differences among the various treatment compounds were both narrow and rather equivalent, with CAP and MET appearing slightly more potent than TAU on the urine $Na^{+,}$ and the reverse been the case on the urine K^+. While these actions were enhanced by pairing CAP and MET with each other or individually with TAU and by treating the diabetic rats with a ternary combination, the gain in potency relative to monotherapy was negligible. Even though the use of an ACE inhibitor like CAT can lead to

an increase in the plasma and a corresponding decrease in the urine of the K^+ level, the present study verified the opposite trend, possibly because of only an incipient renal dysfunction (Riebel et al. 2010). The improved urinary excretion of K^+ and Na^+ by MET probably reflects the enhancing effect of this drug on the glomerular filtration rate (Dorella et al. 1996) which, in turn, may reflect a renoprotective ability against GLC-induced nephropathy, including an antioxidant and antiapoptotic effect at the renal tubular level (Nasri et al. 2013). A similar argument has been advanced to explain the benefits of TAU on diabetic nephropathy (Abebe and Mozaffarin 2011), to which one can add the preservation of the normal renal anatomy and decreased tubular fibrosis (Ito et al. 2012).

Localized tissue oxidative stress is regarded as a key component in the development and progress of DN. A key initiating factor seems to be chronic hyperglycemia, and mitochondrial dysfunction seems to play a key role. Oxidative stress leading to the production of ROS is known to contribute to the oxidation of important biomolecules, including proteins, lipids, carbohydrates and DNA and, hence, to diabetic complications including nephropathy (Forbes et al. 2008). In addition, oxidative stress is regarded as an accelerating factor for the formation of MDA, a LPO end product that can be detected by immunohistochemistry in diabetic renal glomerular lesions and trough chemical tests in the plasma and urine of diabetic patients with glomerulosclerosis and mesangial expansion (Chang et al. 2005). In the present study MDA was clearly elevated in the kidney of diabetic rats, an effect that was effectively reduced to almost basal levels by CAP, MET and TAU and by their combinations. The present results on CAP confirm the results of a previous study indicating that ACE inhibitors like CAP and enalapril can be useful in lowering LPO, measured as MDA, in the diabetic kidney (Kęziora-Kornatowska 1999; Kędziora-Kornatowska et al. 2000). Furthermore, it has been reported that the administration of MET to rats made diabetic with STZ has a profound decreasing effect (almost 50%) on the renal MDA level (Erejuwa et al. 2011). However, these results are in clear contrast with those of a study in newly diagnosed type 2 diabetic patients and in which a 12 weeks treatment with this biguanide did not have a clear effect on the plasma MDA (Gupta et al. 2010). On the other hand, rats made diabetic with SZT and consuming TAU (1%) as part of the drinking water showed a marked reduction of the renal MDA (Wang et al. 2008), and the administration of this amino acid by oral gavage to Otsuka Long-Evans Tokushima Fatty rats resulted in a reduction of the urine MDA (Koh et al. 2014). Although the present study has verified that a treatment of diabetic rats with either CAP, MET or TAU and their various combinations can virtually normalize the renal MDA levels, they are, in all likelihood, operating by different mechanisms, especially since they differ quite widely in their quantitative effects on the circulating levels of GLC and HbA_{1c}. Hence, an earlier proposal suggesting that the occurrence of oxidative stress leading to a higher than normal LPO is closely dependent on the prevailing circulating levels of both GLC and HbA_{1c} (Bandeira et al. 2012) may need a revision, at least in the case of TAU, to include other mechanisms of antioxidant protection. In agreement with the results of the present work, which finds TAU to lower the renal levels of MDA, there is evidence to indicate a difference in response between different animal models of

diabetes since a 1-week consumption of this amino acid as part of the drinking water by KK mice, a genetically hyperglycemic animal model of type 2 diabetes, was able to lower the liver and pancreatic islet cell levels of MDA; but the same treatment approach failed to reproduce this effect in the liver and pancreas of alloxan-treated ICR mice, an animal model of type 1 DM (Lim et al. 1998). These results are illustrative of the variability of the effect of TAU on LPO which, in addition to the type of animal model of diabetes used, is also dependent on the area of the kidney examined (Trachtman et al. 1995).

The renal GSH concentration was moderately decreased (~35%) and that of GSSG was markedly increased (>125%) in diabetic rats. While some investigators have reported decreases of the GSH in the diabetic kidney (Anjaneyulu and Chopra 2004), others have verified no changes in either the GSH (Baştar et al. 1998) or the GSH plus GSSG contents (Bräunlich et al. 1994) in the same animal model of diabetes used here. In the present study CAP, MET and TAU were found to reduce the loss of the renal GSH, an effect that was, however, only significant with MET. On the other hand, combining MET or CAT with TAU raised the ability of these compounds to preserve the renal GSH stores, and providing the diabetic rats with MET-CAP or MET-CAP-TAU led to only a negligible loss of the renal GSH. In the case of GSSG, however, all the three test compounds were able to lower the accumulation of this index of oxidative stress in the diabetic kidney, with TAU appearing much more effective than MET and CAP in that order. A combined treatment with binary combinations of CAP and MET either with TAU or with each other led to a reduction of the diabetic GSSG concentration to level insignificantly different from the control value. On the other hand, a concurrent treatment with the three compounds reduced the diabetic GSSG value to a nearly normal value. Based on these results and on the effects of the test compounds on the redox ratio (GSH/GSSG), it can be inferred that all the test compounds used in the present study can significantly antagonize the lowering effect of diabetes on the ratio. In this case, TAU appeared as the most protective, MET as the least and CAP providing an intermediate protection. In addition, the protective effect of MET was found to increase not only in the presence of TAU but also of CAP.

Another contributing factor to diabetic microvascular disease appears to be a deficiency of antioxidant enzymes such as CAT and SOD, which are known to participate in the respective detoxification of harmful hydrogen peroxide and superoxide anion radical (Sindhu et al. 2004); and of the selenoprotein GPx, which simultaneously reduces organic hydroperoxides to the corresponding alcohols and hydrogen peroxide to water, with GSH serving as a co-substrate (Hamanishi et al. 2004). A review of the scientific literature has disclosed that a wide variation exists in the reported activities of these enzymes in the diabetic kidney, with some studies finding higher than normal activities of GPx and SOD along with a lower of CAT activity (Erejuwa et al. 2011), a decreased CAT and SOD activity plus an increased GPx activity (Wohaieb and Godin 1987), a generalized decrease in the activity of all three enzymes (Sadi et al. 2012) or even no changes (Elmali et al. 2004). Regardless of the trend of the changes in the activities of these enzymatic antioxidant defenses, their deficiencies emerge as an important factor in the etiology of diabetic renal

complications since in diabetes there is an increase in the production of both hydrogen peroxide and lipid peroxides by renal cells and a balance between the production of oxidants, notably superoxide anion and hydrogen peroxide, and the status of antioxidant defenses in the form of antioxidant enzymes determines the extent of the oxidative stress and the occurrence of renal injury (Asaba et al. 2005). In the present study the activities of all three antioxidant enzyme were markedly decreased in the diabetic kidney, by ~70% in the case of CAT and GPx and by more than 80% in the case of SOD, compared to corresponding activities in normal kidneys. Treating the diabetic rats with CAP, MET or TAU raised these values by at least one-half of the diabetic value, effects that were improved to when these compounds were used together, more as a ternary combination than as a binary combination with TAU.

5 Conclusions

This study has verified that in rats made diabetic with STZ, an oral treatment with CAP, MET or TAU and their binary and tertiary combinations can lead to a marked improvement of the metabolic, biochemical and functional alterations associated with the diabetic state, with the differences in potency among the treatment compounds varying according to parameter being evaluated and the treatment approach used, being usually greater when TAU was combined with either MET or CAP or, better, with both compounds. When used on naive rats, the test compounds were usually without significant effects on the parameter evaluated in the present study.

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