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

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

Abbreviations

INS	Insulin
MET	Metformin
STZ	Streptozotocin
TAU	Taurine

1 Introduction

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

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

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

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

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

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

2 Methods

2.1 Animals and Treatments

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

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

2.2 Samples and Assays

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

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

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

2.3 Statistical Analysis of the Data

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

3 Results

3.1 Metabolic Changes

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

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

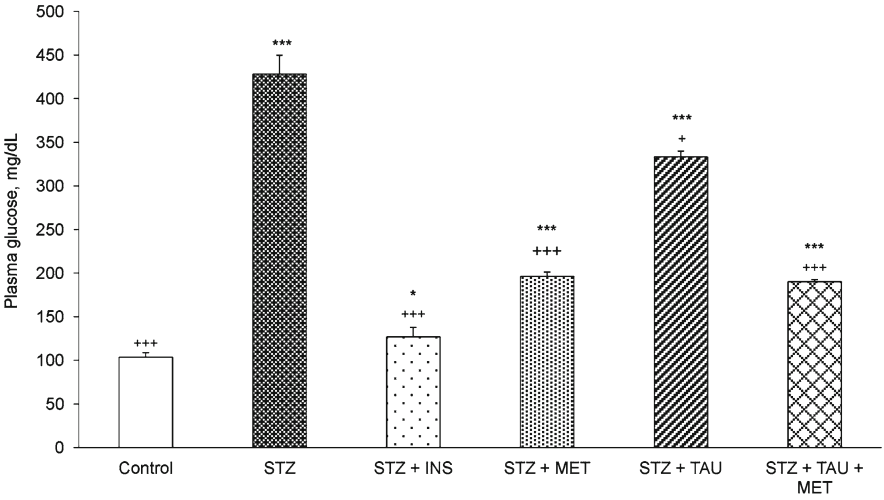

Fig. 1 The effect of treating diabetic (STZ) rats with INS, MET, TAU and MET-TAU, starting 15 days after a STZ dose and continuing for the next 41 days, on the diabetic plasma glucose level. Differences were significant vs. Control at $^{*}p<0.05$ and $^{***}p<0.001$; and vs. STZ at $^{+}p<0.05$ and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

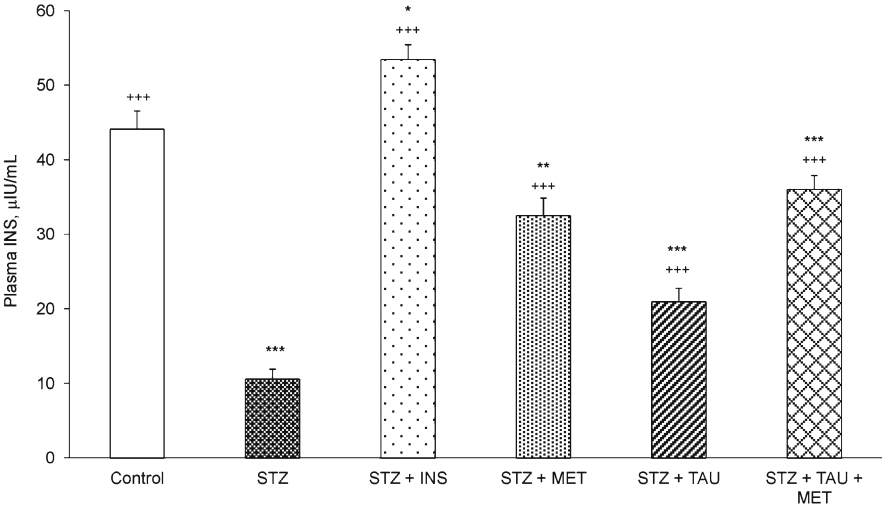

Fig. 2 The effect of treating diabetic (STZ) rats with INS, MET, TAU and MET-TAU, starting 15 days after a STZ dose and continuing for the next 41 days, on the diabetic plasma INS level. Differences were significant vs. Control at $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$; and vs. STZ at $^{+++}p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

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

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

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

3.2 *Oxidative Stress*

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

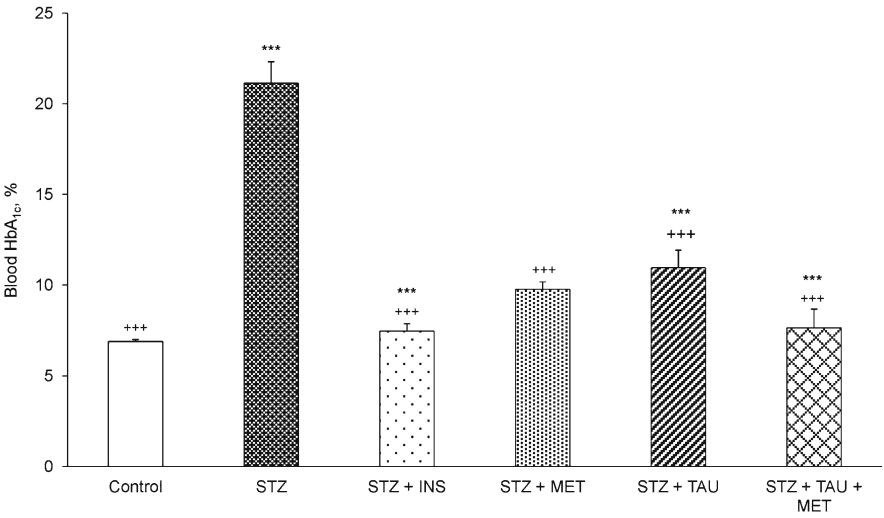

Fig. 3 The effect of treating diabetic (STZ) rats with INS, MET, TAU and MET-TAU, starting 15 days after a STZ dose and continuing for the next 41 days, on the diabetic blood HbA_{1c} level. Differences were significant vs. Control at $^{***}p<0.001$; and vs. STZ at $^{+++}p<0.001$.

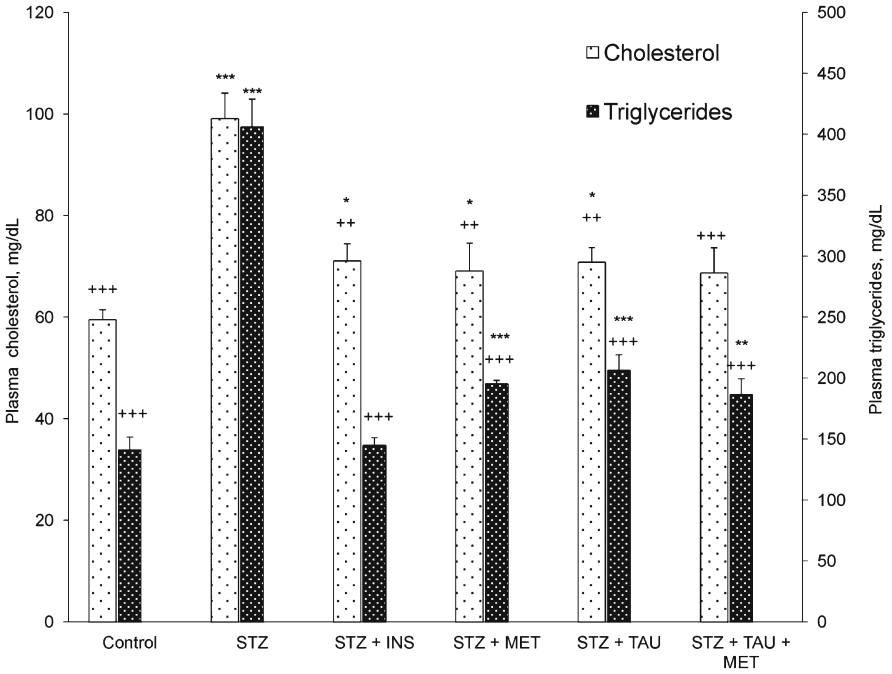

Fig. 4 The effect of treating diabetic (STZ) rats with INS, MET, TAU and MET-TAU, starting 15 days after a STZ dose and continuing for the next 41 days, on the diabetic plasma cholesterol and triglycerides levels. Differences were significant vs. Control at $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$;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

promoted a LPO that reached equivalent extents (41 % and 42 % increases, respectively, $p<0.01$ vs. control) in the plasma and kidney (Fig. 5). A treatment with MET, TAU or their combination was found to virtually abolish MDA formation (only 1–6 % increases). The same degree of protection was attained with an INS treatment (Fig. 5). Diabetes reduced the GSH level in the plasma (by 46 %, $p<0.001$) and kidney (by 34 %, $p<0.01$) together with the corresponding GSH/GSSG ratios (by 75 % in the plasma, by 71 % in the kidney, both at $p<0.001$ vs. controls) (Fig. 6). These changes were effectively counteracted by all the treatment agents ($p\leq0.01$), with TAU and MET-TAU providing the greatest protection, followed by INS and MET.

3.3 Renal Function and Nephropathy

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

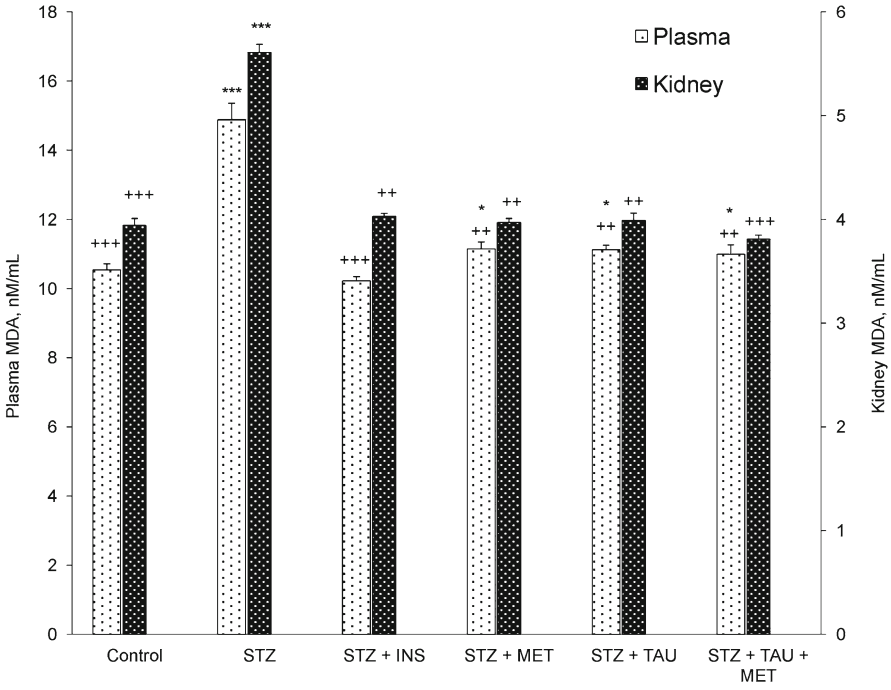

Fig. 5 The effect of treating diabetic (STZ) rats with INS, MET, TAU and MET-TAU, starting 15 days after a STZ dose and continuing for the next 41 days, on the diabetic plasma and kidney MDA levels. Differences were significant vs. Control at $^{*}p<0.05$ and $^{***}p<0.001$; and vs. STZ at $^{++}p<0.01$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

Diabetic rats showed a 5.7-fold increase in the urine output (60 mL/day) over that of normal rats. INS was very effective in reducing this output (only threefold increase), an effect that was shared, although to a lesser degree, by MET, TAU and MET-TAU (4.7-fold, 4.9-fold and 4.6-fold increases, respectively, $p<0.05$ vs. diabetes) (Fig. 7). The massive excretion of proteins in the urine seen in diabetic rats (>150 % increase) was drastically reduced by all the treatment agents ($p\leq0.01$ vs. diabetes), with INS showing the greatest effect (by 60 %), followed by MET (by 49 %) and TAU (by 40 %). The effect of a combined treatment with MET-TAU was intermediate to that seen with the individual compounds (46 % decrease) (Fig. 8). Diabetic rats exhibited significantly higher levels of plasma creatinine and urea nitrogen than control rats (by >330 % and >150 %, respectively, $p<0.001$) (Fig. 9). MET and TAU were able to reduce these changes significantly, with TAU providing a slightly greater protective effect (54 % and 35 % reductions, respectively) than MET (45 % and 22 % reductions, respectively). Relative to the individual treatments, one with TAU plus MET led to a greater effect in the case of the plasma urea nitrogen (52 % reduction) but not in the case of the plasma creatinine (43 % reduction). INS was as effective as TAU in reducing the diabetic plasma creatinine (58 % less, $p<0.001$) and as MET-TAU on the plasma urea nitrogen (49 % less, $p<0.001$) (Fig. 9). Diabetes also affected the plasma levels of Na^{+} and K^{+}, which were found

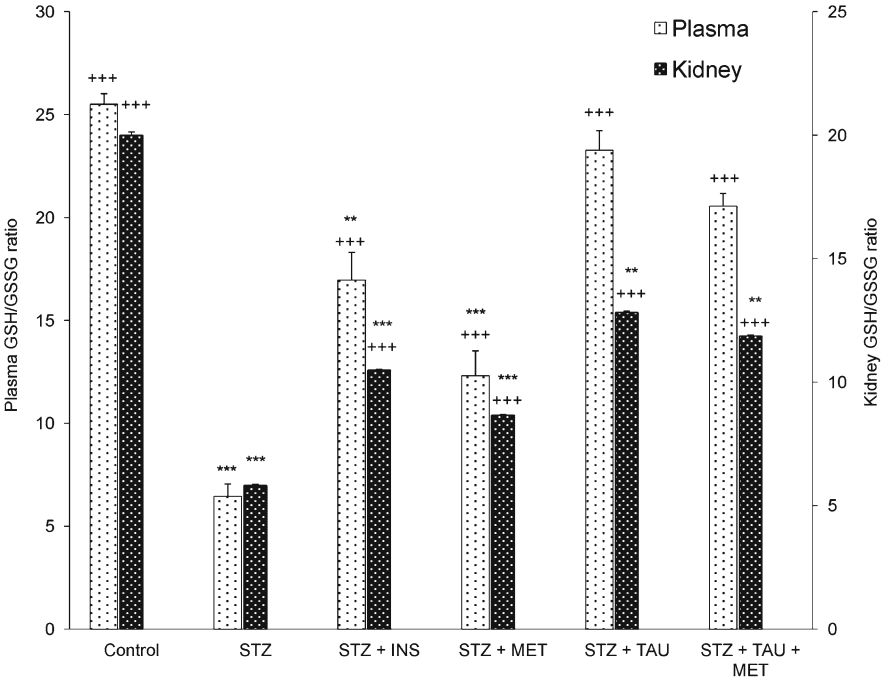

Fig. 6 The effect of treating diabetic (STZ) rats with INS, MET, TAU and MET-TAU, starting 15 days after a STZ dose and continuing for the next 41 days, on the diabetic plasma and kidney GSH/GSSG ratios. Differences were significant vs. Control at $^{**}p<0.01$ and $^{***}p<0.001$; and vs. STZ at $^{+++}p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

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

4 Discussion

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

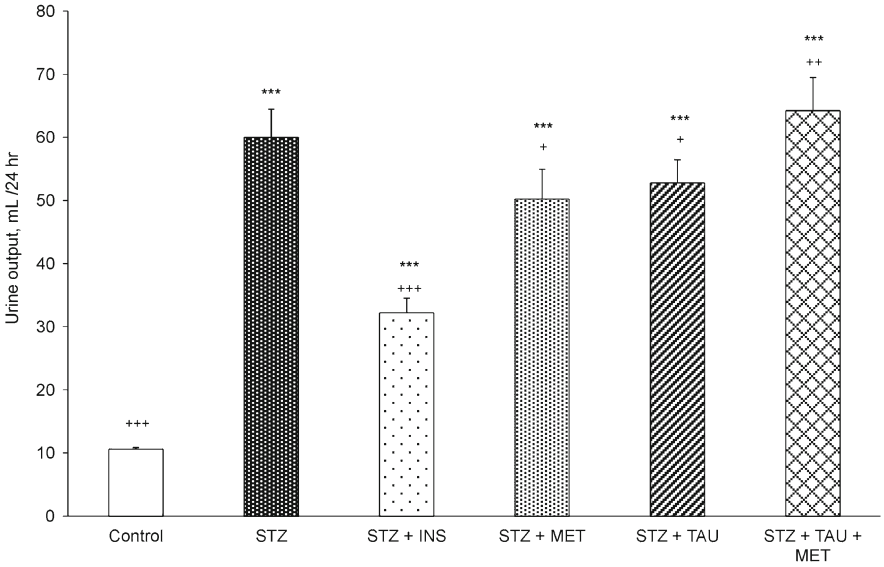

Fig. 7 The effect of treating diabetic (STZ) rats with INS, MET, TAU and MET-TAU, starting 15 days after a STZ dose and continuing for the next 41 days, on the diabetic urinary output. Differences were significant vs. Control at $^{***}p<0.001$; and vs. STZ at $^{+}p<0.05$, $^{++}p<0.01$ and $^{+++}p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

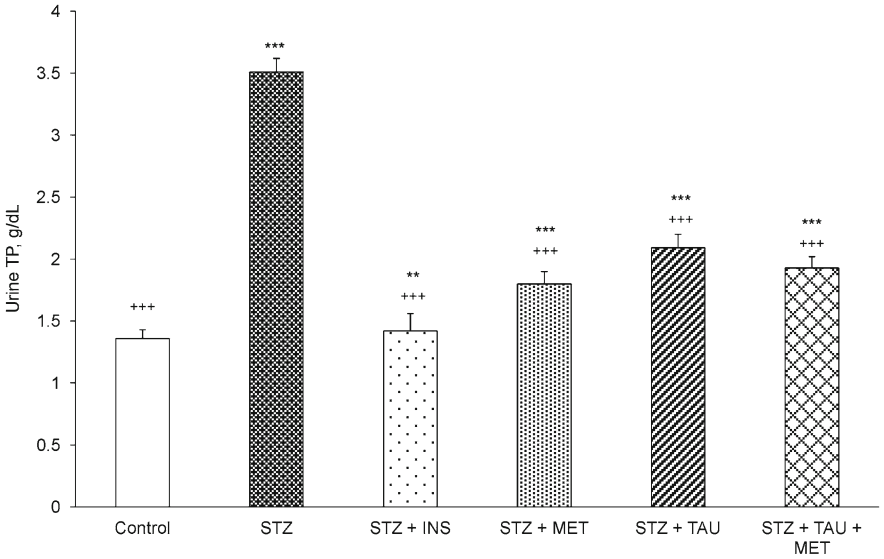

Fig. 8 The effect of treating diabetic (STZ) rats with INS, MET, TAU and MET-TAU, starting 15 days after a STZ dose and continuing for the next 41 days, on the diabetic urine TP level. Differences were significant vs. Control at $^{**}p<0.01$ and $^{***}p<0.001$; and vs. STZ at $^{+++}p<0.001$.

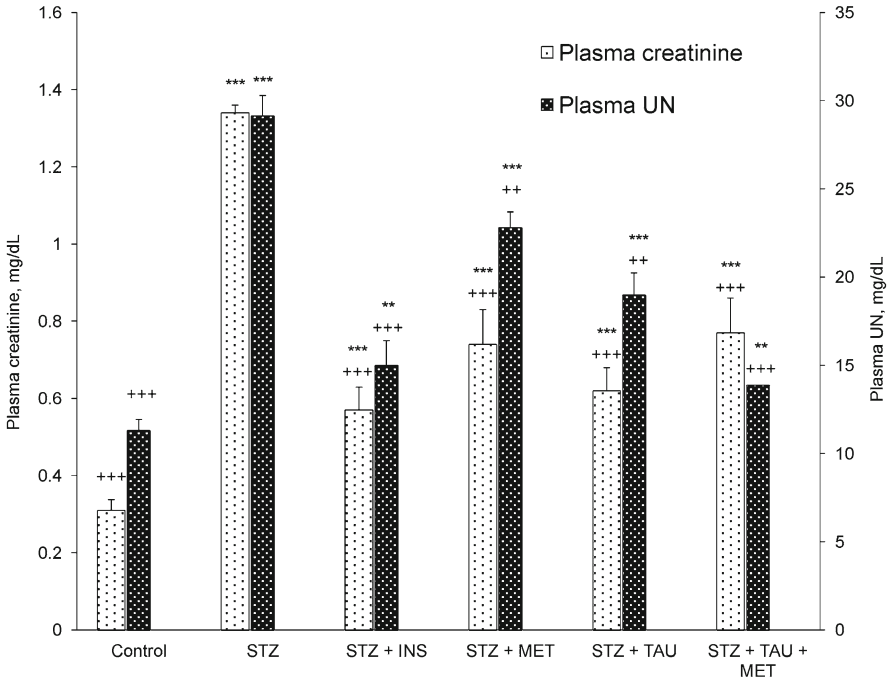

Fig. 9 The effect of treating diabetic (STZ) rats with INS, MET, TAU and MET-TAU, starting 15 days after a STZ dose and continuing for the next 41 days, on the diabetic plasma creatinine and urea nitrogen levels. Differences were significant vs. Control at $^{**}p<0.01$ and $^{***}p<0.001$; and vs. STZ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

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

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

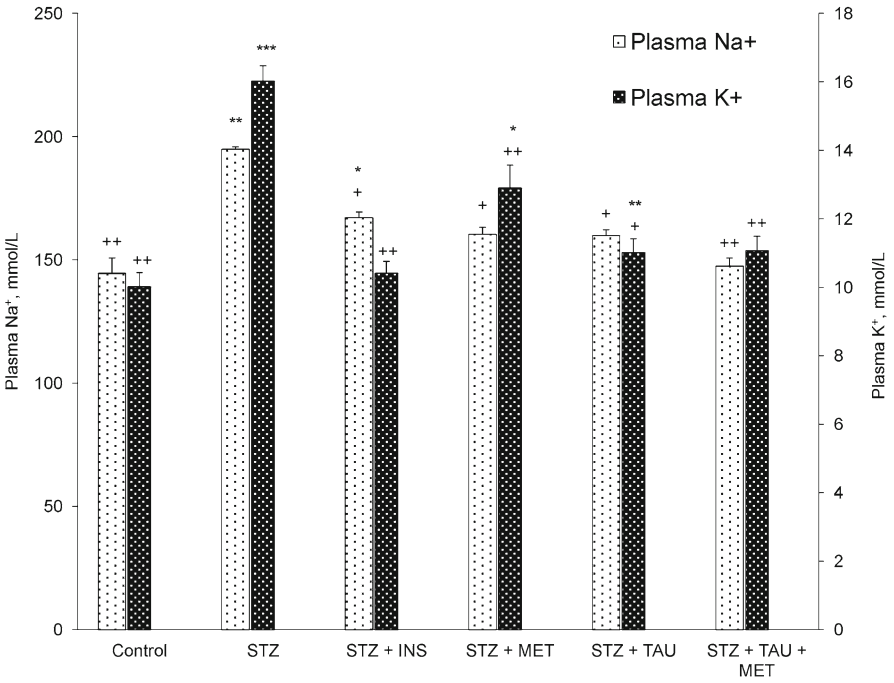

Fig. 10 The effect of treating diabetic (STZ) rats with INS, MET, TAU and MET-TAU, starting 15 days after a STZ dose and continuing for the next 41 days, on the diabetic plasma Na^+ and K^+ levels. Differences were significant vs. Control at $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$; and vs. STZ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

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

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

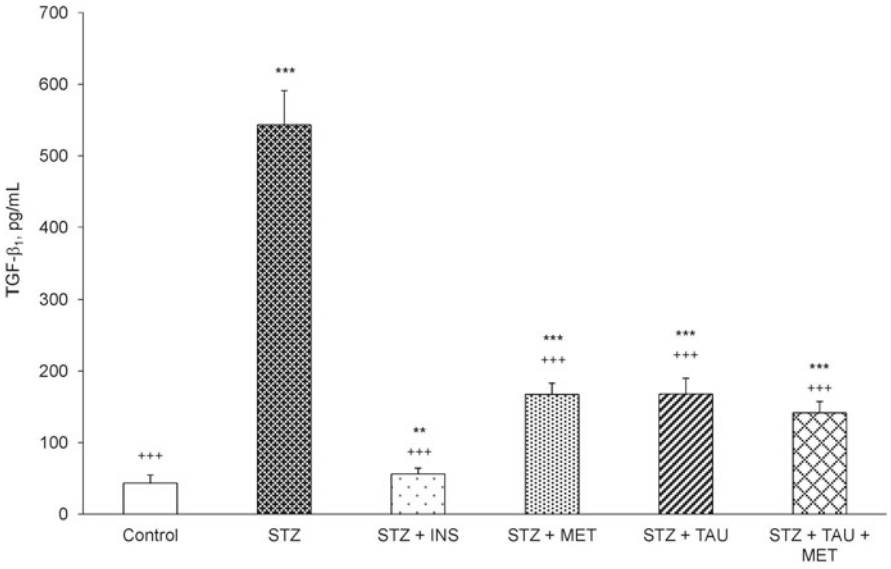

Fig. 11 The effect of treating diabetic (STZ) rats with INS, MET, TAU and MET-TAU, starting 15 days after a STZ dose and continuing for the next 41 days, on the plasma TGF-β1 level. Differences were significant vs. Control at $^{**}p<0.01$ and $^{***}p<0.001$; and vs. STZ at $^{+++}p<0.001$.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

5 Conclusion

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

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

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