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ORIGINAL ARTICLE

Taurine ameliorates alloxan-induced diabetic renal injury, oxidative stress-related signaling pathways and apoptosis in rats

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Abstract Hyperglycemia-induced oxidative stress plays a vital role in the progression of diabetic nephropathy. The renoprotective nature of taurine has also been reported earlier; but little is known about the mechanism of this beneficial action. The present study has, therefore, been carried out to explore in detail the mechanism of the renoprotective effect of taurine under diabetic conditions. Diabetes was induced in rats by alloxan (single i.p. dose of 120 mg/kg body weight) administration. Taurine was administered orally for 3 weeks (1% w/v in drinking water) either from the day on which alloxan was injected or after the onset of diabetes. Alloxan-induced diabetic rats showed a significant increase in plasma glucose, enhanced the levels of renal damage markers, plasma creatinine, urea nitrogen and urinary albumin. Diabetic renal injury was associated with increased kidney weight to body weight ratio and glomerular hypertrophy. Moreover, it increased the productions of reactive oxygen species, enhanced lipid peroxidation and protein carbonylation in association with decreased intracellular antioxidant defense in the kidney tissue. In addition, hyperglycemia enhanced the levels of proinflammatory cytokins (TNF-α, IL-6, IL-1β) and Na^+–K^+-ATPase activity with a concomitant reduction in NO content and eNOS expression in diabetic kidney. Investigation of the oxidative stress-responsive signaling cascades showed the upregulation of PKCα, PKCβ, PKCε and MAPkinases in the renal tissue of the diabetic animals. However, taurine administration decreased the elevated blood glucose and proinflammatory cytokine levels, reduced renal oxidative stress (via decrease in xanthine oxidase activity, AGEs formation and inhibition of p47phox/CYP2E1 pathways), improved renal function and protected renal tissue from alloxan-induced apoptosis via the regulation of Bcl-2 family and caspase-9/3 proteins. Taurine supplementation in regular diet could, therefore, be beneficial to regulate diabetes-associated renal complications.

Keywords Alloxan · Apoptotis · Hyperglycemia · Kidney · Mitochondria · Renoprotection · Taurine

Abbreviations

ALX	Alloxan
CAT	Catalase
DAB	3,3′-Diaminobenzidine tetrahydrochloride
FACS	Fluorescence activated cell sorting
GSH	Glutathione
GSSG	Glutathione disulfide
GST	Glutathione S-transferase
GPx	Glutathione peroxidase
GR	Glutathione reductase
IL-6	Interleukin-6
MDA	Malonaldehyde
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TAU	Taurine
TNF-α	Tumor necrosis factor alpha

Introduction

Diabetic nephropathy, one of the most serious complications of both type 1 and type 2 diabetes mellitus, is a major cause of end-stage renal disease. At early stage, diabetic

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nephropathy has no symptoms but it develops in late stage as a result of increased excretion of proteins in the urine or due to renal failure (Ibrahim and Hostetter 1997). Literatures suggest that about 50% of the patients suffering from diabetes mellitus for more than 10 years develop complications from diabetic nephropathy (Yan et al. 2007; Barr 2000). Kidney does not depend on insulin for glucose uptake; thus the increased plasma glucose levels in diabetes will also result in high intracellular levels of glucose and face severe and sustained hyperglycemia (Chandra et al. 2002). Evidence suggests that hyperglycemia is associated with increased production of reactive oxygen species (ROS) and the resulting oxidative stress is thought to play a key role in the pathogenesis of this disorder (Winiarska et al. 2009). The possible enzymatic and non-enzymatic sources of ROS in the diabetic kidney are autoxidation of glucose, transition metal-catalyzed Fenton reactions, advanced glycation end products (AGEs), polyol pathway flux, mitochondrial respiratory chain deficiencies, xanthine oxidase activity, peroxidases, nitric oxide synthase (NOS) and NAD(P)H oxidase (Forbes et al. 2008). Therefore, an agent having both glycemic control and antioxidant potentials might be considered protective against diabetic kidney damage (Manna et al. 2009a; Palsamy and Subramanian 2011).

Taurine (2-aminoethanesulfonic acid), a non-protein amino acid, is found in millimolar concentration in mammalian tissues and possess cytoprotective properties in a variety of tissues. The source of taurine in the body is its biosynthesis from methionine and cysteine mainly in the liver and dietary intake. Taurine modulates a variety of cellular functions, including antioxidation, ion movement, osmoregulation, modulation of neurotransmitters, conjugation of bile acids and membrane stabilization, regulates intracellular Ca^{2+} concentration, inhibits apoptosis and reduces the levels of pro-inflammatory cytokines (Aerts and Van Assche 2002; Das et al. 2008, 2010a, b, 2011a, b, c; Ghosh et al. 2009; Kontny et al. 2000; Manna et al. 2008a, b, 2009b; Racasan et al. 2004; Roy et al. 2009; Roy and Sil 2012; Sinha et al. 2007, 2008a, b, 2009). Taurine inhibits nephrotoxicity, renal cell death (both apoptosis and necrosis), oxidative stress via increasing the activities of antioxidant enzymes and intracellular GSH and nitrosative stress, normalizes the Na^+–K^+-ATPase activity and reduced TNF-α as well as NO production in kidney during various toxin and drug-induced pathophysiological conditions (Das et al. 2010c; Roy et al. 2009; Manna et al. 2009b). The anti-hyperglycemic and nephroprotective roles of taurine in diabetes have already been reported (Winiarska et al. 2009; Das et al. 2011a; Franconi et al. 2006; Gavrovskaya et al. 2008). Besides, very limited in vivo and in vitro studies have demonstrated that the protective effect of taurine on diabetic kidney is mediated via suppression of AGEs, TGFβ and ROS (Winiarska et al. 2009; Nandhini and Anuradha 2003; Higo et al. 2008). Therefore, the present study has been undertaken to investigate the possible mechanisms of beneficial action of taurine against alloxan-induced diabetic renal complications in rats. Alloxan (2,4,5,6-tetraoxypyrimidine; 2,4,5,6-pyrimidinetetrone) is widely used to induce experimental diabetes in animals. It destroys the pancreatic β cells via ROS generation and causes IDDM or insulin-dependent diabetes mellitus. The present study describes the mechanism of the prophylactic role of taurine on hyperglycemia-mediated renal dysfunction, oxidative stress and ROS-mediated signaling pathways (PKCs and MAPKinases), proinflammatory cytokines and NOS expressions in alloxan-induced diabetic rats. The outcome of the present study might provide important information and mechanistic insight into its usefulness as a therapeutic agent for the treatment of diabetes and associated renal injury.

Materials and methods

Chemicals

Taurine (2-aminoethane sulfonic acid), alloxan, bovine serum albumin (BSA), bradford reagent, anti-Bcl-2, anti-Bcl-XL, anti-Bad and anti-Bax antibodies were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Other antibodies like anti-PKCα, anti-PKCβ, etc. were purchased from Abcam (UK). Kits for measurement of blood glucose, LDH, uric acids and total cholesterol were purchased from Span Diagnostic Ltd., India. All other chemicals were bought from Sisco Research Laboratory, India.

Animals

Adult male Wister rats weighing approximately 160–180 g were purchased from M/S Gosh Enterprises, Kolkata, India. Animals were acclimatized under laboratory conditions for 2 weeks prior to experiments. They were maintained under standard conditions of temperature (23 ± 2°C) and humidity (50 ± 10%) with an alternating 12 h light/dark cycles. The animals had free access to tap water and fed standard pellet diet (Agro Corporation Private Ltd., Bangalore, India). All the experiments with animals were carried out according to the guidelines of the Institutional Animal Ethical Committee (IAEC), Bose Institute, Kolkata (the permit number is IAEC/BI/3(I) cert./2010) and full details of the study was approved by both IAEC and Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), Ministry of Environment & Forests, New Delhi, India (the permit number is 95/99/CPCSEA).

Experimental design for in vivo treatments

Experimental design needed for the present in vivo study has been summarized as follows: rats were randomly assigned to five groups and treated as follows:

Group 1 Normal group: rats received neither alloxan nor taurine, received vehicle only.
Group 2 TAU group: rats received only 1% (w/v in water) taurine orally.
Group 3 ALX group: rats received single dose of alloxan (ALX, 120 mg/kg body weight with citrate buffer, pH 4.5, i.p.) (Verma et al. 2010). Alloxan-injected rats with blood glucose level in excess of 300 mg/dL, 3 days after the injection were considered as diabetic.
Group 4 ALX&TAU simultaneous treatment group: rats received taurine (orally, 1% w/v in water, 1 h before ALX injection) from the day on which alloxan was injected until the 21st day.
Group 5 ALX+TAU post treatment group: rats received taurine (orally, 1% w/v in water) from the 4th day after alloxan injection until 24th day.

After the experiment periods (treatment for 3 weeks with taurine), animals were killed under light ether anesthesia and kidneys were collected.

Collection of blood, urine and kidney

The rats were killed after the experimental period and the kidneys were removed and stored at −80°C till later analysis. The body weight and kidney weight were measured and compared between groups. Blood samples were drawn from the caudal vena cava, collected in test tubes containing heparin solution and centrifuged at 1,500*g* for 10 min to obtain plasma. The plasma was immediately stored at −80°C until use. Urine for albumin determination was withdrawn from bladder immediately after animal killing and stored at −80°C until use.

Preparation of mitochondrial, cytosolic and microsomal fractions

The kidneys were minced, washed with saline buffer and homogenized in a Dounce glass homogenizer in homogenizing buffer (50 mM phosphate buffer/1 mM EDTA, pH 7.5, containing 1.5 mM $MgCl_2$, 10 mM KCl, and supplemented with protease and phosphatase inhibitors). The homogenates were spun down for 10 min at 2,000*g* at 4°C. The pellet was discarded and the supernatant was recentrifuged at 12,000*g* for 10 min at 4°C. The pellet was resuspended in 200 mM mannitol, 50 mM sucrose, 10 mmol/L Hepes–KOH (pH 7.4) and stored as mitochondrial fraction at −80°C. The final supernatant was further centrifuged at 105,000*g* for 60 min at 4°C. The resulting microsomal pellets were then suspended in a 0.25 mM sucrose solution containing 1 mM EDTA and stored at −80°C until use. The supernatant was taken and used as cytosolic fraction and stored at 4°C.

Determination of protein content

The protein content of the experimental samples was measured by the method of Bradford (1976) using crystalline BSA as standard.

Determination of renal dysfunction markers

Plasma glucose and specific markers related to renal dysfunction in the plasma and urinary albumin were estimated using standard kits.

Histological studies

Kidneys from the normal and experimental rats were fixed in 10% buffered formalin and were processed for paraffin sectioning. Sections of about 5 μm thickness were stained with hematoxylin and eosin to evaluate under light microscope. The average volume of the glomeruli was measured. Mean values were calculated from each of six glomeruli per section. Histologic scoring system was used to quantitate renal pathology. The scoring system was as follows: 0, absent; 1, present and 2, marked. The parameters assessed were glomerulosclerosis and tubulointerstitial damage.

Plasma/renal taurine level detection

Plasma/renal taurine level was measured according to the method of Ferreira et al. (1997). In brief, sulphosalicylic acid solution was added to the kidney homogenate or plasma and allowed to stand for 10 min. Then it was filtered through W42 paper and derivatised with *o*-phthalaldehyde and 2-mercaptoethanol. The derivative was then analyzed by HPLC using an UV absorbance detector at 350 nm.

Biochemical analyses

The lipid peroxidation, protein carbonyl content and intracellular ROS were estimated following the method described by Das et al. (2009). Activities of antioxidant enzymes (SOD, CAT, GST, GR) and cellular metabolites levels (GSH and GSSG) in the renal tissue were determined following the method described by Das et al. (2009).

Determination of xanthine oxidase and Na^+–K^+-ATPase activities

Xanthine oxidase activity was assessed by measuring the enzymatic oxidation of xanthine. The reactive mixture contained 1.9 mL of 50 mM potassium phosphate buffer, pH 7.5 and 1 mL of 0.15 mM xanthine. The reaction was started by adding 100 μL of tissue extract and the increase in absorbance was measured at 290 nm for 4 min. The Na^+–K^+-ATPase activity was measured following the method of Das et al. (2010a, b, c).

Determination of plasma AGEs and renal TNF-α, IL-6 and IL-1β levels by ELISA

The levels of plasma AGEs and renal TNF-α, IL-6 and IL-1β levels were determined by ELISA kits according to the manufacturer's instructions (Abcam, UK).

Determination of renal hydroxyproline level

The kidney hydroxyproline levels were estimated according to the method of Woessner (1961).

Determination of renal nitric oxide production

Nitric oxide (NO) decomposes quickly in aerated solutions to produce stable nitrite/nitrate products. The renal NO level was indirectly assessed by measuring the nitrite levels in cytosolic fraction using a colorimetric method based on the Griess reaction (Green et al. 1982; Suresh and Das 2006). Twenty microliters of the analyzed sample was added to 100 μL of the Griess reagent (equal volumes of 2% sulfanilamide in 5% HCl and 0.1% N^1-(1-naphthyl), N^2-diethylethylenediamine oxalate in H_2O were mixed just before use), NADH and nitrate reductase. One thousand microliters H_2O was added, the mixture was stirred, and, after 5 min, the absorbance at 540 nm was determined. The nitrite level in the cytosolic fraction was determined using the standard curve, which was constructed using sodium nitrite as the standard.

Determination of mitochondrial membrane potential ($\Delta\psi_m$)

Analytic flow cytometric measurements for the membrane potential ($\Delta\psi_m$) of isolated mitochondria were performed using a FACScan flow cytometer with an argon laser excitation at 488 and a 525-nm band-pass filter. Mitochondrial membrane potential ($\Delta\psi_m$) was estimated on the basis of cell retention of the fluorescent cationic probe rhodamine 123.

Immunoblotting

An equal amount of protein (50 μg) from each sample was resolved by 10% SDS-PAGE and transferred to PVDF membrane. Membranes were blocked at room temperature for 2 h in blocking buffer containing 5% non-fat dry milk to prevent non-specific binding and then incubated with anti-eNOS (1:1,000 dilution), anti-iNOS (1:1,000 dilution), anti-nNOS (1:1,000 dilution), anti-ERK (1:1,000 dilution), anti-JNK (1:1,000 dilution), anti-p38 (1:1,000 dilution), anti-PKCα (1:250 dilution), anti-PKCβ (1:250 dilution), anti-Bad (1:1,000 dilution), anti-Bax (1:1,000 dilution), anti-Bcl-2 (1:1,000 dilution), anti-Bcl-xL (1:1,000 dilution), anti-cytochrome *c* (1:1,000 dilution), anti-caspase-3 and anti-caspase-9 (1:100 dilution) primary antibodies separately at 4°C overnight. The membranes were washed in TBST (50 mmol/L Tris–HCl, pH 7.6, 150 mmol/L NaCl, 0.1% Tween 20) for 30 min and incubated with appropriate HRP-conjugated secondary antibody (1:2,000 dilution) for 2 h at room temperature and developed by the HRP substrate 3,3′-diaminobenzidine tetrahydrochloride (DAB) system (Bangalore Genei, India).

DNA fragmentation assay

Renal tissue was washed with STE buffer (0.1 M NaCl, 10 mM Tris–HCl, 1 mM EDTA, pH 8) and 1 mL homogenization buffer (0.1 M NaCl, 0.2 M sucrose, 0.01 M EDTA, 0.3 M Tris, pH 8), and 100 μL 10% SDS was added and mixed by vortexing and incubated at 65°C for 1 h. 175 μL of 8(M) potassium acetate was then added and incubated in ice for 1 h, centrifuged and the supernatant was collected. Equal volume of phenol–chloroform mixture was added, mixed and centrifuged to separate phases. The upper-most layer was taken in a fresh tube. Equal volume of chloroform was added and centrifuged. The aqueous layer was taken in a fresh tube. 1/10th vol. of 3(M) sodium acetate (pH 7.4) and 2.5 times vol. of ethanol were added and centrifuged. The precipitated DNA was washed with 80% ethanol.

The DNA fragmentation has been assayed by electrophoresing genomic DNA samples, isolated as described above from normal as well as experimental rat kidneys on agarose/EtBr gel.

Statistical analysis

All the values are expressed as mean ± SD ($n = 6$). Significant differences between the groups were determined with SPSS 10.0 software (SPSS Inc., Chicago, IL, USA) for Windows using one-way analysis of variance (ANOVA), and the group means were compared by

Duncan's multiple range test (DMRT). A difference was considered significant at the $P < 0.05$ level.

Results

Taurine repressed the change in kidney and body weight, water intake, plasma glucose and nephrotoxicity

Among the ALX injected rats, 85% developed experimental IDDM as characterized by decreased body weight (29%) and increased kidney weight (94%), increased water consumption (475%) and increased plasma glucose (335%) (Table 1). Approximately 50% of the ALX-injected rats died. However, treatment with taurine (1%) for 3 weeks increased the body weight (35%), decreased the kidney weight (39%) and the plasma glucose (41%) and increased the survival rate (70–80%) significantly in rats suffering from ALX-induced diabetes. ALX-induced nephrotoxicity was also evident from the increased kidney weight to the body weight ratio (172%) and increased levels of plasma BUN (181%), creatinine (271%), uric acid (100%) and urinary albumin (208%), which are markers of diabetic nephropathy (Table 1). However, taurine treatment effectively reduced the kidney weight to the body weight ratio (56%), levels of plasma BUN (58%), creatinine (57%), uric acid (46%) and urinary albumin (42%) in diabetic rats.

Histological assessment

Glomerular injury and glomerular hypertrophy are the prominent features of diabetic nephropathy. Sections from ALX-unexposed animals showed normal histology (histological score 0). In the ALX-induced diabetes rats, after 3 weeks there was an increase in the glomerular volume/hypertrophy (35%, Table 1; Fig. 1), glomerulosclerosis (describing scarring of the glomeruli in kidneys; histological score 2.58) and tubulointerstitial damage as evident from numerous damaged and degenerated tubules (histological score 2.55) (Fig. 1). Glomerulosclerosis and tubulointerstitial damage are directly associated with the progression of diabetic nephropathy. Scarring of glomeruli disturbs the filtering process of the kidneys and allows protein to leak from the blood into urine (proteinuria). Tubulointerstitial damage occurs due to a complex interplay between factors in the tubular epithelial cells, tubular lumen, peritubular capillaries, resident and infiltrating interstitial cells and extracellular matrix (Harris 2001). However, treatment with taurine significantly reduced glomerular volume/hypertrophy (15%), glomerulosclerosis (histological score 0.14) and tubulointerstitial (histological score 0.13) damage in the diabetic animals.

Diabetes reduced the plasma/renal taurine levels

In the diabetic rats, the plasma/renal taurine levels were found to be significantly lower (35 and 41%, respectively) than that of normal rats (Table 1). Taurine treatment of normal rats resulted in significantly higher plasma/renal taurine (230 and 48%, respectively) compared with normal rats (Table 1). Its levels in the "taurine-simultaneous" (170 and 28%, respectively) and "taurine-post treated" (177 and 30%, respectively) groups were found to be even higher compared with the taurine alone treated rats (Table 1).

Table 1 Effect of alloxan and taurine on the kidney weight, body weight, glomerular volume, water intake, plasma glucose, levels of the markers related to renal dysfunction and plasma/renal taurine levels in experimental animals

Parameters	Normal control	TAU treated	ALX treated	ALX&TAU	ALX+TAU
Body weight (g)	204.4 ± 7.11	205.2 ± 7.56	145.6 ± 5.16[a]	197.8 ± 6.91[b]	194.0 ± 6.93[b]
Kidney weight (g)	0.51 ± 0.02	0.55 ± 0.02	0.99 ± 0.04[a]	0.59 ± 0.03[b]	0.63 ± 0.03[b]
Ratio of the kidney weight to the body weight (%)	0.25 ± 0.012	0.27 ± 0.012	0.68 ± 0.031[a]	0.29 ± 0.013[b]	0.32 ± 0.013[b]
Glomerular volume (μm^3, $\times 10^3$)	2.41 ± 0.15	2.43 ± 0.17	3.24 ± 0.23[a]	2.81 ± 0.11[b]	2.74 ± 0.13[b]
Water intake (mL/day)	28 ± 1.11	36 ± 1.12	161 ± 6.05[a]	168 ± 7.13[a]	170 ± 7.52[a]
Plasma glucose (mg/dL)	107 ± 8	97 ± 7	465 ± 12[a]	265 ± 12[b]	285 ± 10[b]
Creatinine (mg/dL)	0.42 ± 0.02	0.40 ± 0.02	1.56 ± 0.07[a]	0.65 ± 0.03[b]	0.71 ± 0.03[b]
BUN (mg/dL)	16.1 ± 0.66	15.48 ± 0.62	62.56 ± 2.68[a]	24.53 ± 1.06[b]	28.38 ± 1.13[b]
Uric acid (mg/dL)	5.14 ± 0.22	5.16 ± 0.28	10.31 ± 0.33[a]	5.52 ± 0.18[b]	5.64 ± 0.18[b]
Urinary Albumin (mg/dL)	3.7 ± 0.19	3.6 ± 0.20	11.4 ± 0.48[a]	6.5 ± 0.26[b]	6.8 ± 0.28[b]
Plasma taurine (μM)	26 ± 1.11	86 ± 3.32[a]	17 ± 0.68[a]	233 ± 9.65[a,b]	238 ± 9.94[a,b]
Renal taurine (μmol/g tissue)	28.49 ± 1.11	43.12 ± 1.84[a]	16.8 ± 0.62[a]	54.94 ± 2.15[a,b]	55.46 ± 2.42[a,b]

Values are expressed as mean ± SD, for six animals in each group

[a] Values differ significantly from normal ($P < 0.05$)

[b] Values differ significantly from ALX ($P < 0.05$)

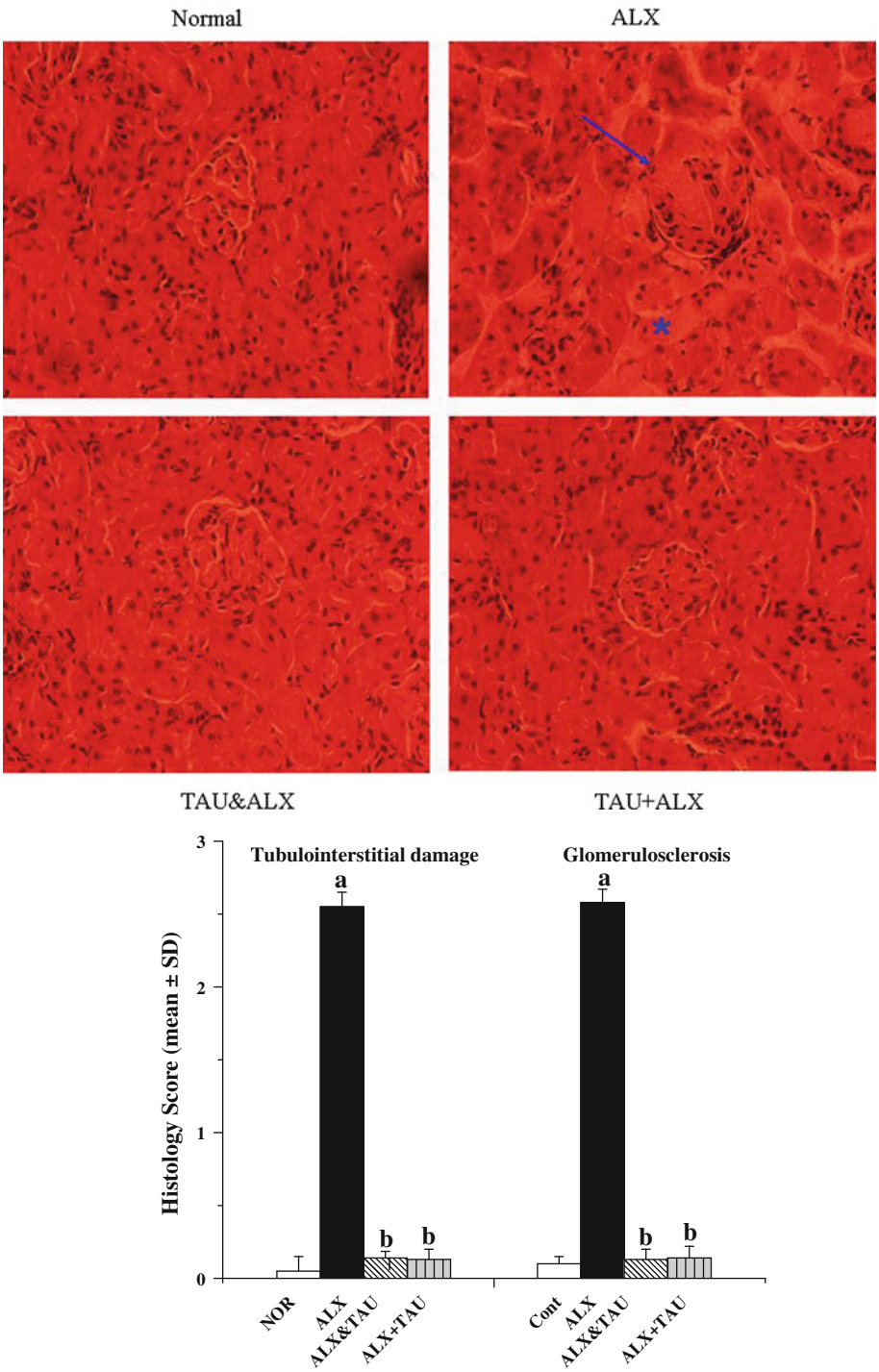

Fig. 1 Hematoxylin and eosin stained sections of kidney from normal, ALX-treated (single i.p. dose of 120 mg/kg body weight) and taurine-treated (1% w/v in water, orally for 3 weeks) animals. Normal animals showed normal appearance of glomeruli; ALX-treated animals showed glomerular hypertrophy, glomerulosclerosis (marked with *arrow*) and tubulointerstitial damage (marked with *asterisk*); taurine-treated animals showed almost normal appearance of glomeruli (×10). Histologic scoring system was used to quantitate renal pathology. The scoring system was as follows: 0, absent; 1, present and 2, marked. The parameters assessed were glomerulosclerosis and tubulointerstitial damage. Data represent the average ± SD of six separate experiments in each group. *a* the significant difference between the control and ALX-exposed groups and *b* the significant difference between ALX+TAU/ ALX&TAU treated and ALX-exposed groups ($^{a}P < 0.05$, $^{b}P < 0.05$)

Suppressing effect of taurine on ALX-induced oxidative stress in diabetic rat kidney

In the present study, we observed decreased GSH:GSSG ratio (60%) and anti-oxidant enzymes activities; SOD (82%), CAT (45%), GST (73%) and GR (68%) in the kidney of diabetic rats compared with normal ones. The elevation of intracellular GSSG was not significant (13%), whereas diabetes reduced the GSH/GSSG ratio as a result of decreased GSH content (55%) (Table 2). On the other hand, intracellular ROS level (114%) (Table 2), lipid peroxidation (209%) and protein carbonylation (233%) (Table 2) were increased. All these above alterations clearly reflected the involvement of oxidative stress in this present pathophysiological condition. However, treatment with taurine significantly increased GSH:GSSG ratio (112%), anti-oxidant enzymes activities; SOD (350%), CAT (58%), GST (223%), GR (236%) and decreased lipid

Table 2 Effect of alloxan and taurine on the status of intracellular thiol-based antioxidants, intracellular ROS, activities of the antioxidant enzymes, lipid peroxidation and protein carbonylation in renal tissue

Parameters	Normal control	TAU treated	ALX treated	ALX&TAU	ALX+TAU
GSH (nmol/mg protein)	10.57 ± 0.47	10.49 ± 0.45	4.76 ± 0.24[a]	5.41 ± 0.25[a]	5.29 ± 0.22[a]
GSSG (nmol/mg protein)	0.37 ± 0.02	0.35 ± 0.02	0.42 ± 0.03	0.22 ± 0.02[a,b]	0.23 ± 0.02[a,b]
Redox ratio (GSH/GSSG)	28.57 ± 1.34	29.97 ± 1.35	11.33 ± 0.52[a]	24.59 ± 1.26[b]	23.00 ± 1.24[b]
Intracellular ROS (% over control)	100 ± 4.1	71 ± 2.45	214 ± 9.8[a]	108 ± 4.2[b]	116 ± 4.4[b]
SOD (U/mg protein)	20.34 ± 1.48	22.58 ± 1.47	3.57 ± 0.43[a]	18.48 ± 1.27[b]	14.81 ± 1.17[b]
CAT (μmol/min/mg protein)	115.36 ± 5.39	121.69 ± 9.75	63.38 ±3.72[a]	104.34 ± 5.25[b]	96.81 ± 4.55[b]
GST (μmol/min/mg protein)	0.62 ± 0.03	0.69 ± 0.03	0.17 ± 0.02[a]	0.57 ± 0.03[b]	0.53 ± 0.03[b]
GR (nmol/min/mg protein)	30.89 ± 1.69	32.99 ± 1.58	9.74 ± 0.56[a]	33.45 ± 1.76[b]	32.78 ± 1.74[b]
MDA (nmol/mg protein)	3.18 ± 0.19	3.17 ± 0.20	9.83 ± 0.65[a]	4.51 ± 0.32[b]	4.76 ± 0.33[b]
Protein carbonylation (nmol/mg protein)	4.28 ± 0.23	4.23 ± 0.21	14.28 ± 0.16[a]	9.57 ± 0.63[b]	9.73 ± 0.64[b]

Values are expressed as mean ± SD, for six animals in each group

[a] Values differ significantly from normal ($P < 0.05$)

[b] Values differ significantly from ALX ($P < 0.05$)

Table 3 Effect of alloxan and taurine on the xanthine oxidase activity, plasma AGEs level and Na^+–K^+-ATPase enzymes activity

Name of the enzymes	Normal control	TAU treated	ALX treated	ALX&TAU	ALX+TAU
Xanthine oxidase activity (mU/mg protein)	0.31 ± 0.03	0.27 ± 0.03	0.53 ± 0.06[a]	0.33 ± 0.05[b]	0.35 ± 0.04[b]
Plasma AGEs (μg/mL)	487.58 ± 18.12	480.62 ± 18.40	958.28 ± 35.27[a]	724.58 ± 25.28[b]	748.59 ± 26.85[b]
Na^+–K^+ -ATPase (μmol/min/mg protein)	4.27 ± 0.24	4.18 ± 0.22	7.85 ± 0.54[a]	5.29 ± 0.26[b]	5.37 ± 0.27[b]

Values are expressed as mean ± SD, for six animals in each group

[a] Values differ significantly from control ($P < 0.05$)

[b] Values differ significantly from ALX ($P < 0.05$)

peroxidation (53%), protein carbonylation (33%) and ROS level (49%) compared with ALX-exposed group and thereby proving taurine to be an effective anti-oxidant in this regard. Taurine supplementation did not prevent the diabetes-induced decrease in renal GSH level (11%) but significantly decreased GSSG level (48%) and normalized the GSH/GSSG ratio.

Taurine reduced the activity of xanthine oxidase and expressions of p47phox/CYP2E1 in diabetic kidney

In our present study, we observed an increased activity of xanthine oxidase (71%), an important biological source of ROS in diabetic kidney (Table 3). Cytosolic ROS production can result from the activation of NADPH oxidase. Western blot analysis showed that the p47phox cytosolic component of NADPH oxidase was increased in diabetic kidney (Fig. 2). ALX also increased the renal expression of CYP2E1, another important ROS inducer under diabetic conditions (Fig. 2). However, taurine suppressed these increased activities of xanthine oxidase (38%) and p47phox/CYP2E1 expressions, indicating that taurine could reduce the ROS production in diabetic kidney via suppression of p47phox/CYP2E1 and xanthine oxidase activity.

Taurine normalized the plasma AGEs level and Na^+–K^+-ATPase activity in diabetic kidney

Since AGEs play a key role in the development of diabetic complications, such as nephropathy, we have investigated

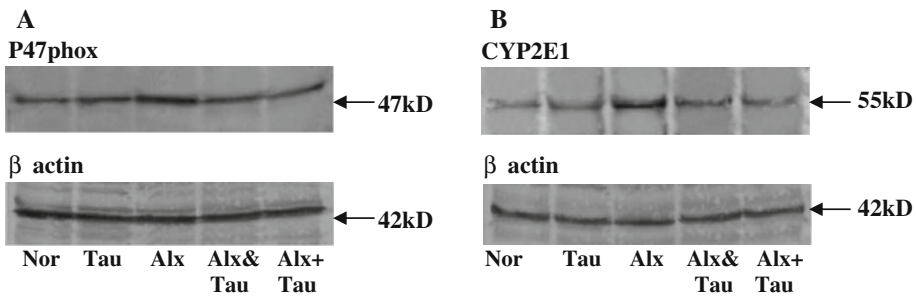

Fig. 2 Effect of taurine (1% w/v in water, orally for 3 weeks) and ALX (single i.p. dose of 120 mg/kg body weight) on p47phox and CYP2E1 protein expressions. **a** p47phox and **b** CYP2E1. Taurine reduced the ALX-induced increased expressions of both p47phox and CYP2E1

whether taurine could be able to inhibit its formation. In our experimental animals, the plasma AGEs level was 487.58 μg/mL and Na^+–K^+-ATPase activity was 4.27 μmol/min/mg protein in normal condition. The diabetic group of rats showed a significant increase in plasma AGEs level (97%) and Na^+–K^+-ATPase activity (84%) compared with normal ones (Table 3). However, taurine treatment reduced the AGEs level (24%) and Na^+–K^+-ATPase enzyme activity (33%) in diabetic animals.

Taurine declined proinflammatory cytokines in diabetic kidney

The effect of taurine on the levels of TNF-α, IL-1β and IL-6 in renal tissues of normal and experimental groups of rats was investigated. The levels of TNF-α, IL-1β (data not shown) and IL-6 (Fig. 3a) were escalated significantly in ALX-induced diabetic rats in comparison with normal control. However, treatment with taurine significantly altered these levels to near normalcy when compared with diabetic group.

Taurine lowered the hydroxyproline levels in the kidneys of diabetic rats

Collagen is considered as a dependable marker of fibrosis and usually determined by estimating the hydroxyproline content. Figure 3b shows that renal hydroxyproline levels in ALX-induced diabetic rats were significantly higher compared with normal ones. However, in rats receiving taurine simultaneously or post to hyperglycemia, hydroxyproline levels were markedly lower than in diabetic animals.

Taurine suppressed the activation of PKCs and MAPkinases in diabetic kidney

Activation of PKCs and MAPKs are associated with some of the pathophysiological changes associated with diabetic nephropathy. Immunoblot analysis shows (Fig. 4a–f) that ALX induction stimulated the expression of PKCα, PKCβ, PKCε and several MAPkinases, e.g. phospho-p38, phospho-ERK1/2 and phospho-JNK. However, treatment with taurine down-regulated the expressions of PKCs and MAPkinases induced by ALX.

Effect of taurine on the NO production and NOS expressions in diabetic kidney

In the ALX-induced diabetic animals, there was significant decrease in renal expression of eNOS protein and NO contents compared with the normal animals (Fig. 5). However, ALX did not significantly alter the expressions of iNOS and nNOS. The ALX-induced decrease in the eNOS protein level and renal NO contents were restored to the normal levels by treatment with taurine.

Taurine protected against ALX-induced apoptosis in kidney

ALX-induced renal cell death in diabetic rats was investigated via Western blot analyses and DNA gel electrophoresis. Administration of ALX induced a clear apoptotic response as indicated by increased protein expressions of cleaved caspase-9/3 and the ladder pattern obtained in DNA gel electrophoresis (Fig. 6). Further, we have investigated the involvement of intrinsic apoptotic pathway and observed that ALX significantly increased the pro-apoptotic

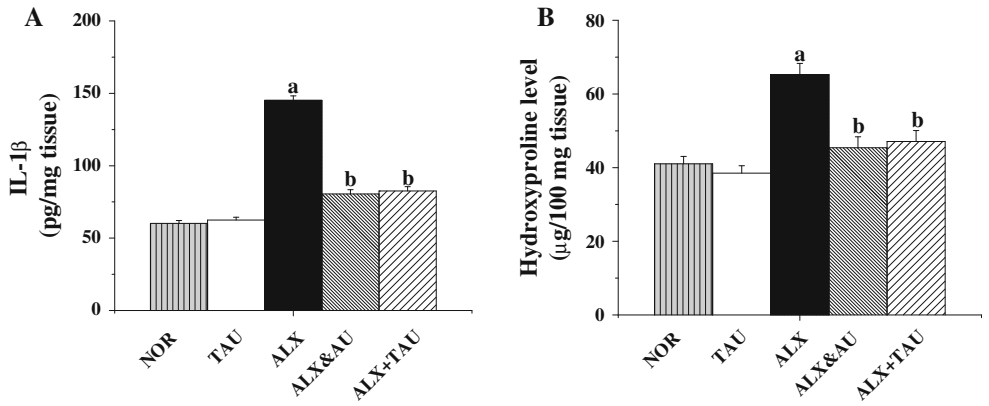

Fig. 3 Effect of taurine (1% w/v in water, orally for 3 weeks) and ALX (single i.p. dose of 120 mg/kg body weight) on the levels of proinflammatory cytokine and hydroxyproline after 3 weeks of treatment. **a** IL-1β and **b** hydroxyproline. Taurine reduced the ALX-induced increased levels of proinflammatory cytokines and hydroxyproline. Data represent the average ± SD of six separate experiments in each group. *a* the significant difference between the control and ALX-exposed groups and *b* the significant difference between ALX+TAU/ALX&TAU treated and ALX-exposed groups ($^aP < 0.05$, $^bP < 0.05$)

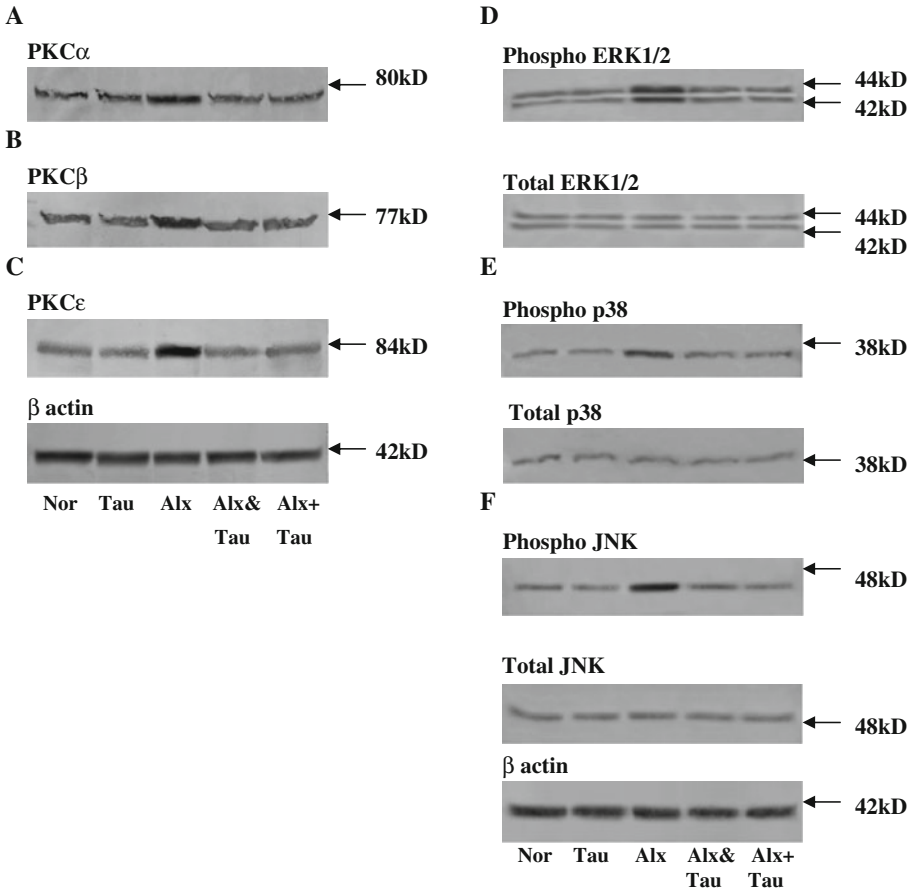

Fig. 4 Effect of taurine (1% w/v in water, orally for 3 weeks) and ALX (single i.p. dose of 120 mg/kg body weight) on PKCα, PKC*β*, PKCε and mitogen activated protein kinases (MAPKs). **a** PKCα, **b** PKC*β*, **c** PKCε, **d** phospho and total ERK1/2, **e** phospho and total p38 and **f** phospho and total JNK. Taurine reduced the ALX-induced increased expressions of all PKC isoforms and MAPKs

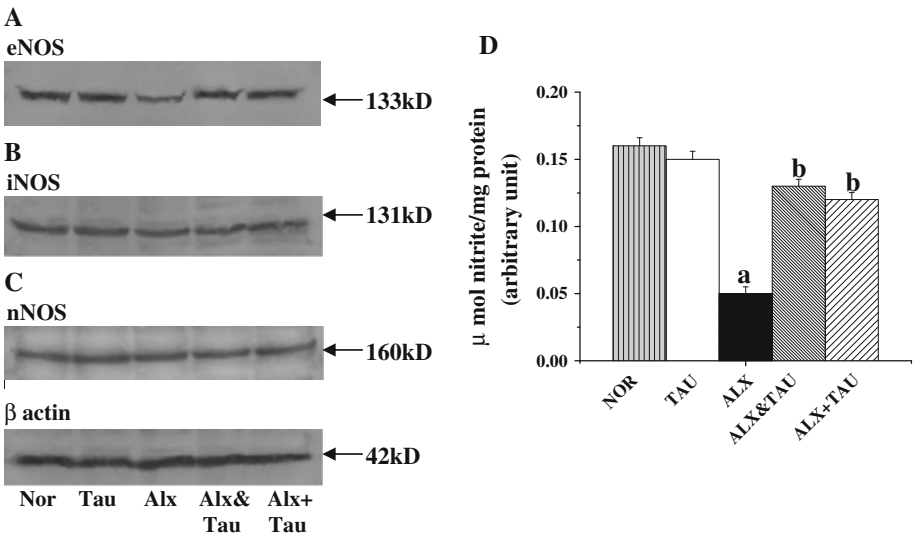

Fig. 5 Effect of taurine (1% w/v in water, orally for 3 weeks) and ALX (single i.p. dose of 120 mg/kg body weight) on renal nitric oxide (NO) content and the protein expressions of eNOS, iNOS and nNOS. **a** eNOS, **b** iNOS, **c** nNOS and **d** renal NO content. Taurine increased the ALX-induced decreased expression of eNOS and NO level. Data represent the average ± SD of six separate experiments in each group. *a* the significant difference between the control and ALX-exposed groups and *b* the significant difference between ALX+TAU/ALX&TAU treated and ALX-exposed groups ($^{a}P < 0.05$, $^{b}P < 0.05$)

Bad/Bax and decreased the anti-apoptotic Bcl-2/Bcl-xL protein expressions (Fig. 6). In addition, ALX also reduced the mitochondrial membrane potential and increased the translocation of mitochondrial cytochrome *c* into cytosol (Fig. 6). However, taurine effectively reduced the ALX-induced cleavage of caspase-9/3, translocation of

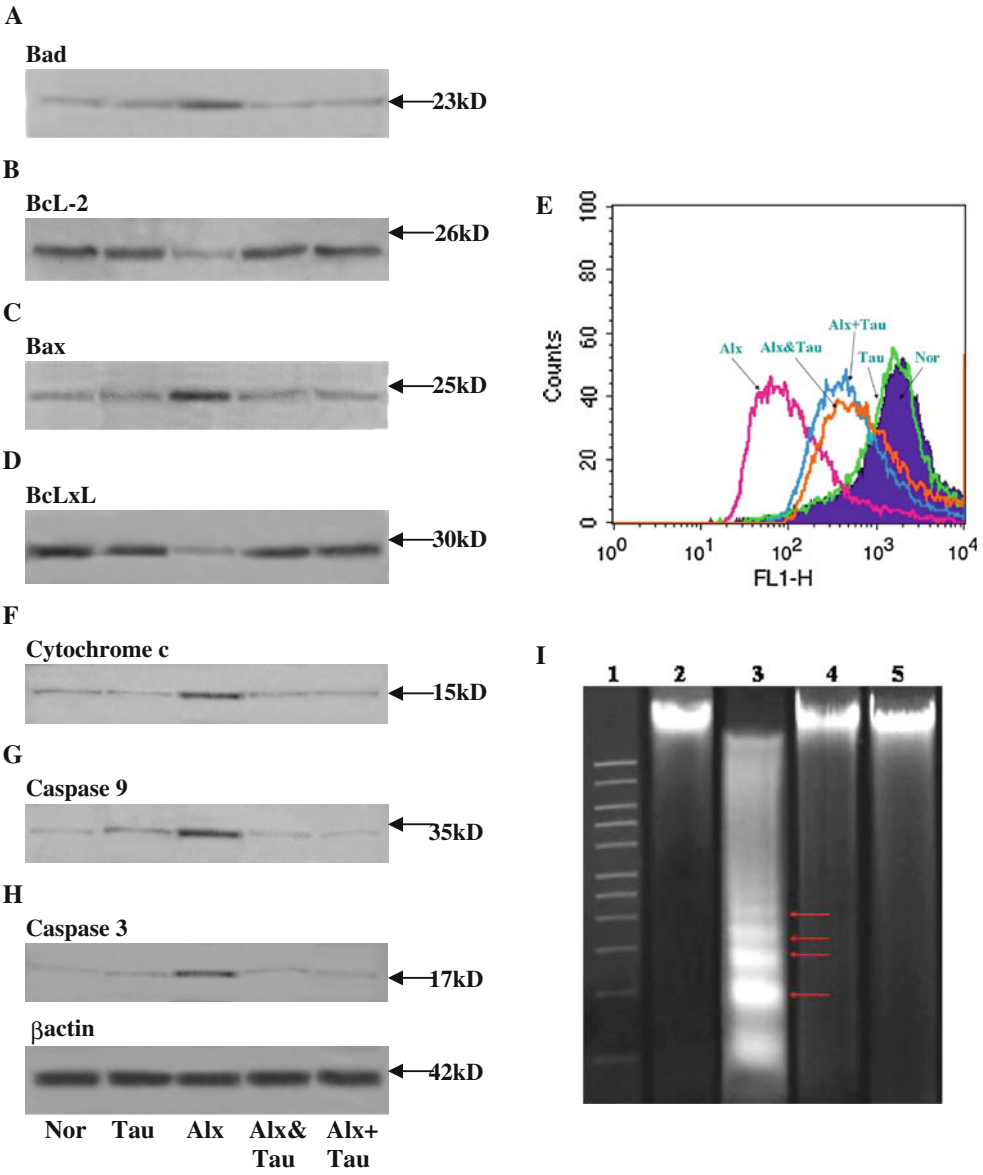

Fig. 6 Effect of taurine (1% w/v in water, orally for 3 weeks) and ALX (single i.p. dose of 120 mg/kg body weight) on renal apoptosis. **a** Bad, **b** Bcl-2, **c** Bax, **d** Bcl-xL, **e** mitochondrial membrane potential, **f** cytochrome *c*, **g** caspase-9, **h** caspase-3 and **i** DNA fragmentation pattern on agarose/EtBr gel. *Lane 1* marker (1 kb DNA ladder), *lanes 2, 3, 4 and 5* DNA isolated from normal, ALX intoxicated, taurine simultaneous and post treated rats, respectively. *Arrows* indicate ladder formation. Taurine decreased all the ALX-induced proapoptotic events in renal tissue

cytochrome *c* into cytosol and increase in the expressions of pro-apoptotic Bad/Bax. On the other hand, taurine increased the expressions of anti-apoptotic Bcl-2/Bcl-xL, mitochondrial membrane potential and DNA integrity in renal tissues of ALX-exposed rats. All these results clearly indicated the anti-apoptotic role of taurine in renal tissue under ALX-induced diabetic conditions.

Discussion

Hyperglycemia causes oxidative stress and helps developing diabetic complications. Therefore, the ideal antidiabetic drug should possess both hypoglycemic and antioxidative properties. Our present experimental studies suggest that taurine fulfils both of these criteria. In addition, we have investigated the mechanism of antioxidant, anti-inflammatory and anti-apoptotic action of taurine in diabetic kidney.

In the present study, we have chosen 120 mg/kg body weight dose of alloxan, because at this dose, pancreatic β cells were extensively damaged, resulting in significant increase in blood glucose levels due to less insulin secretion (Verma et al. 2010). We showed that taurine significantly decreased plasma glucose levels in ALX-induced diabetic rats and ameliorated the increase in kidney weight to body weight ratio. Similar hypoglycemic effects of taurine have also been observed in rodent models (Winiarska et al. 2009; Gavrovskaya et al. 2008). However, several other studies have also reported no changes in plasma glucose levels in taurine-treated diabetic rats (Obrosova et al. 2001; Li et al. 2005). This contradiction

might result due to many factors, including the mode of taurine administration. It is clear that taurine would be more effectively absorbed if administered through drinking water than in food stuff (Winiarska et al. 2009). This hypoglycemic effect of taurine is mediated possibly via protection of β-cells/β-cell insulin secretion (Gavrovskaya et al. 2008; Chang and Kwon 2000; Kaplan et al. 2004; Cherif et al. 1998), enhanced insulin sensitivity (Wu et al. 2010; Carneiroa et al. 2009; Colivicchi et al. 2004), insulin like effect (Kulakowski and Maturo 1984; Maturo and Kulakowski 1988), diminished glucose absorption from gastrointestinal tract (Kim et al. 2006) and its accelerated utilization by peripheral tissues (Nandhini et al. 2004). ALX also significantly increased the renal damage markers such as, plasma levels of BUN, creatinine, uric acid, urinary albumin and glomerular hypertrophy. In our experiment, taurine showed a significant reduction of the elevated levels of these renal damage markers and glomerular hypertrophy, thereby proving it to be an effective renoprotectant under diabetic conditions.

Interplay between taurine and blood glucose levels seems to be present in diabetes. Plasma taurine concentration in patients and streptozotocin or alloxan-induced diabetic animals with IDDM was significantly lower than in respective controls (Franconi et al. 1995, 1996; Trachtman et al. 1995). We also observed a lower plasma/renal level of taurine in the diabetic rats, which was in good agreement with previous reports. However, taurine treatment to diabetic rats significantly increased the plasma taurine levels compared with "taurine-alone" treated rats. The significantly higher water intake of the diabetic group may have contributed, at least in part, to this effect.

Hyperglycemia causes oxidative stress by generation of excessive ROS, which are responsible for the development of diabetic complications, such as nephropathy (Winiarska et al. 2009; Manna et al. 2009a, b; Baynes and Thorpe 1999). In our present study, the inexorable generation of ROS and lipid peroxides during diabetes-mediated oxidative stress could be correlated to decline in the levels of non-enzymatic antioxidant such as GSH, activities of antioxidant enzymes (SOD, CAT, GST and GR) and increase in the levels of MDA, protein carbonylation and altered the redox ratio of GSH/GSSG. However, taurine administration to diabetic group of rats notably declined the levels of renal lipid peroxidation, protein carbonyls and restored the activities of antioxidant enzymes. SOD dismutases superoxide to H_2O_2, whereas CAT detoxifies H_2O_2 to H_2O, thereby protecting the tissues from highly reactive OH· (Szkudelski 2001). This increased activity of SOD and CAT due to taurine treatment helps to avoid the toxic effects of the free radicals generated during diabetes. In the kidneys of diabetic rats, taurine increased the GSH/GSSG ratio via the reduction of GSSG content, which seems to result from the elevated GR activity without affecting diabetes-induced decrease in renal GSH level. ALX also significantly increased the activity of xanthine oxidase, which is another important biological source of ROS under diabetic conditions (Forbes et al. 2008; Akhileshwar et al. 2007). Superoxide anions derived from NADPH oxidase contributes to the development of diabetic nephropathy (Toba et al. 2009; Onozato et al. 2002). Therefore, we have checked the expression of p47phox, a component of NADPH oxidase and observed that p47phox protein level was increased in the ALX-induced diabetic rat kidney. CYP2E1 is involved in ROS production and oxygen-mediated tissue injury under diabetic conditions (Raza et al. 2004). Immunoblot analysis shows that the level of the microsomal CYP2E1 in the diabetic kidney was increased. However, taurine significantly decreased the activity of xanthine oxidase and expressions of p47phox and CYP2E1, thereby reducing the occurrence of oxidative stress and its associated complications.

During diabetes mellitus, elevations in blood glucose levels contribute to the formation of AGEs and production of free radicals. AGEs also produce ROS (Forbes et al. 2008) and both are associated with cell death and renal dysfunction. Hyperglycemia activates the polyol pathway and produce fructose from glucose. The fructose and its metabolites as well as glucose are involved in the non-enzymatic glycation of cellular proteins. The resulting Schiff base undergoes a structural rearrangement to form Amadori products which further produce α-dicarbonyl compounds and produce AGEs via crosslinking with other proteins (Palsamy and Subramanian 2011; Schaffer et al. 2009). However, taurine could probably reduce the plasma AGEs level in ALX-induced diabetic rats by functioning as a glycation scavenger (Nandhini et al. 2004; Hansen 2001) and reducing the blood glucose levels. A marked increase in the activity of Na^+–K^+-ATPase was observed in the diabetic kidney probably because of an adaptation of nephrons to maintain electrolyte homeostasis in diabetes in face of the increased glomerular filtration rate (GFR) and osmotic diuresis (Siddiqui et al. 2006). Treatment of the diabetic animals with taurine regulated the altered level of Na^+–K^+-ATPase.

Inflammation, proinflammatory cytokines and renal tubule fibrosis play an important role in the development and progression of diabetic nephropathy (Palsamy and Subramanian 2011; Toba et al. 2009; Thomson et al. 2001). In our present study, we observed a significant increase in the levels of TNF-α, IL-1β, IL-6 and hydroxyproline in renal tissue of diabetic animals; this increase reflects the severity of the kidney lesions and inflammation/fibrosis of diabetic rats. However, taurine significantly attenuated these proinflammatory cytokines and hydroxyproline levels, thereby moderating the consequences of inflammation and lowered kidney fibrosis in diabetic rats.

Nitric oxide (NO) is involved in various aspects of renal vascular control and function. Literature suggests that chronic NO inhibition with pharmacological inhibitor of NO synthase (NOS) leads to progressive hypertension and severe renal injury (Baylis et al. 1992). Increased production of ROS and decreased concentrations of NO were reported in both diabetic animals and patients (type-1 and type-2) (Hiramatsu and Arimori 1980; Mohan and Das 1997; Hattori et al. 1991). In the present study, we demonstrated that ALX significantly reduced the renal NO content and eNOS expression without changing the expressions of iNOS and nNOS much. This low level of NO could be due to the inactivation of eNOS by enhanced free-radical generation (Laight et al. 1998; Das 2001). However, treatment with taurine recovered the ALX-induced decrease in NO and eNOS expression in the diabetic rat kidney.

Next we have investigated the involvement of ROS-regulated signaling pathways in diabetic renal dysfunction. ROS-induced activation of PKC and MAPkinases play an important role in the development of diabetic nephropathy (Lee et al. 2003). It has been reported that PKC also increased the ROS generation in diabetic kidney (Lee et al. 2003). In our present study, we observed that ALX upregulated the expression of PKCα, PKCβ, PKCε, phospho-p38, phospho-ERK1/2 and phospho-JNK. Treatment with taurine, however, ameliorated this ROS-induced upregulation of PKC and MAPkinases.

Finally, we have investigated the involvement of ALX-mediated intrinsic apoptotic cell death pathway in renal tissues. In our study, we observed that ALX increased the expressions of pro-apoptotic proteins, such as Bax/Bad and decreased the expressions of anti-apoptotic proteins, such as Bcl-2/Bcl-xL, reduced the mitochondrial membrane potential, increased the release of cytochrome *c* into cytosol and the cleavage of caspase-9/3. Moreover, the DNA, isolated from the kidneys of ALX-administered rats, showed a ladder pattern. All these clearly demonstrated the involvement of mitochondria-dependent apoptotic renal cell death. However, taurine significantly reduced all these proapoptotic events leading to renal cell death.

In the present study, taurine has been administered either simultaneously with ALX or after the onset of diabetes (post treatment) to check whether taurine is equally effective in the renoprotection under diabetic conditions. We observed that in both cases taurine exerted its renoprotective effects without making significant difference in the measured parameters.

Thus, the present study demonstrates the renoprotective nature of taurine in ALX-induced diabetic rats and provides evidence that the protective effects, possibly, by controlling blood glucose level, reduce oxidative stress and proinflammatory cytokine production by the renal tissues. Taurine treatment to ALX-induced diabetic rats exhibits a significant ameliorative potential by attenuating the

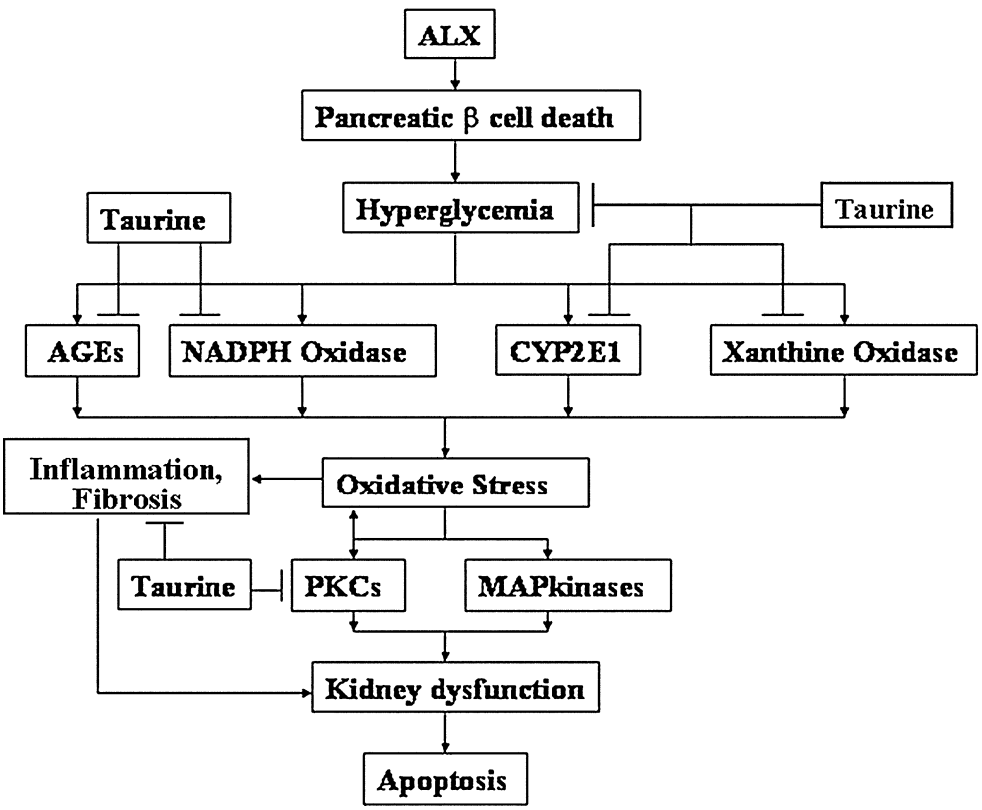

Fig. 7 Schematic diagram of the ALX-induced diabetes and its prevention/curation by taurine

hyperglycemia-mediated oxidative stress via suppression of p47phox/CYP2E1, decrease in AGEs production and xanthine oxidase activity. Moreover, taurine reduces the severity of renal dysfunction via inhibition of ROS-induced activation of PKC and MAPkinases and protects renal tissues against apoptosis induced by ALX (as indicated in the proposed scheme of Fig. 7). Being an endogenous substance in the body, taurine is not associated with toxicity or drug dependence and is well tolerated when used clinically (Chang et al. 2004). Taken together, our results suggest that taurine supplementation may find clinical application to treat renal dysfunction in diabetic patients.

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Conflict of interest The authors have declared that no conflict of interest exists.

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