Taurine Administration after Appearance of Proteinuria Retards Progression of Diabetic Nephropathy in Rats

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Oxidative stress has been postulated to be involved in the development of diabetic nephropathy. In the present study, we evaluated the effect of taurine, an endogenous antioxidant, on diabetic nephropathy by mixing it with the daily drinking water (1% w/v) of streptozotocin-induced diabetic rats from the beginning of the fourth month after the induction of diabetes, during which the urinary protein excretion in untreated diabetic rats showed significant increase in comparison with nondiabetic rats. The taurine administration significantly suppressed further increase in urinary protein excretion in diabetic rats, accompanied by the reduction of mesangial extracellular matrix expansion and TGF-β expression in the renal glomerulus. Immunohistochemical study showed that taurine administration suppressed the intensified stainings to the three different types of oxidative stress markers, such as 8-hydroxyl-2'-deoxyguanosine (8-OHdG), pentosidine, and nitrotyrosine observed in the renal tissues of untreated diabetic rats. These findings suggest that taurine has the ability to suppress the progression of diabetic nephropathy at least in part by its antioxidant property. Since this beneficial effect of taurine was obtained even if its administration was started after the time point when urinary protein excretion already became apparently higher than that of age-matched nondiabetic animals, taurine administration was potentially expected to be applied in clinical field to retard the development of nephropathy in diagnosed diabetic patients.

The severity of diabetic nephropathy is one of the major factors determining the prognosis of diabetic patients. Several lines of evidence have indicated that hyperglycemia causes diabetic nephropathy through a variety of mechanisms, including the induction of oxidative stress (13) and the acceleration of glycation reaction. In fact, various oxidative stress markers, including 8-hydroxydeoxyguanosine (8-OHdG) and nitrotyrosine, have been demonstrated to accumulate in the renal tissues of diabetic animals (12) and humans (27). On the other hand, it has also been postulated that formation of advanced glycation endproducts (AGEs) in the renal tissues is involved in the pathogenesis of diabetic nephropathy (16, 25). Especially, the glycoxidation products, such as pentosidine, are reported to accumulate in the renal glomerulus of diabetic subjects (26).

Local oxidative stress has been postulated to play an important role in the establishment of diabetic glomerular lesions by up-regulation of TGF-β, that accelerates extracellular

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matrix (ECM) production in the glomerulus (17). Therefore, several experimental trials have been performed to suppress the development of diabetic nephropathy through the attenuation of local oxidative stress in the kidneys of diabetic animals. For example, we have proved that overexpression of thioredoxin1, an endogenous antioxidative protein, successfully prevent the development of diabetic nephropathy accompanied by suppression of ECM production (14). However, such gene therapy is hardly applicable in the clinical field at present.

We therefore paid attention to taurine (2-aminoethanesulphonic acid), since it has been orally administered to both animals and humans without significant adverse effects. Taurine is a ubiquitous β-amino acid and is considered as an endogenous antioxidant (6, 8, 33). Recent studies have suggested that taurine potentially protects renal tissues against damage from excess oxidative stress (1, 4, 13, 28, 29). Trachtman et al. (29) showed marked reduction of proteinuria in diabetic rats with decreased lipid peroxidation in the kidney by oral supplementation of taurine from the point when diabetes was induced by streptozotocin (STZ) injection. In the present study, we investigated whether this beneficial effect of taurine on diabetic nephropathy was reproduced even when treatment was started at the time point when urinary protein excretion already showed significant increase in comparison with non-diabetic animals. In addition, we evaluated the effect of taurine treatment on oxidative stress to clarify the relationship between oxidative stress and pathological changes, as well as expression of TGF-β in the glomeruli of diabetic animals.

MATERIALS AND METHODS

Materials

Taurine was purchased from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals were also of analytical grade and purchased from Wako Pure Chemical Industries, unless otherwise noted.

Animals

All animal experiments were conducted according to the Guidelines for Animal Experimentation at Kobe University School of Medicine. Male Sprague Dawley rats (8 weeks old) were obtained from CLEA Japan, Inc. (Osaka, Japan) and randomly divided into three groups as follows: control (n = 6), diabetic rats (n = 6), and taurine-treated diabetic rats (n = 6). Diabetes was induced by a single intravenous injection of STZ (60 mg/kg body weight) freshly dissolved in 200 μl of sterile citrate buffer (pH4.5). Only rats with plasma glucose level over 20 mM one week after STZ treatment were included in the study. Animals were provided with water *ad libitum* and fed a standard laboratory chow. Taurine was added to the drinking water (1 % w/v) for rats of the treatment group. The administration of taurine was started at the beginning of the fourth month after the induction of diabetes, since we found a significant increase in urinary protein excretion rate in the diabetic rats in comparison with control rats at the end of the third month. Fasting blood glucose level was determined by the glucose oxidase method using Glutest Sensor and Glutest Ace (Sanwa Kagaku Kenkyusyo, Nagoya, Japan) (31). Glycated hemoglobin was determined with DCA2000 analyzer (Bayer Medical, Tokyo, Japan). Blood urea nitrogen (BUN) and serum creatinine levels were also measured using an autoanalyzer (Hitachi Model 7700, Tokyo, Japan), immediately before starting taurine-treatment and at the end of animal experiment. Rats were placed in individual metabolic cages once a month, and 24 h urine samples were collected to determine the excreted protein by the method of Bradford (3). Rats had free access to water while in the metabolic cages. Urinary protein excretion rate was corrected by body weight.

Histological study and morphometric analyses

After 7 months of the induction of diabetes, the rats were sacrificed under ether anesthesia. The kidneys were dissected and fixed with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.2) for 48 hours at room temperature, dehydrated through a series of graded ethanol and embedded in paraffin. The 5-mm-thick sections were stained with periodic acid-Schiff (PAS) and periodic acid-methenamine (PAM). Thirty glomeruli were randomly selected from each rat. Evaluation of the mesangial matrix expansion was performed as described elsewhere (23). In brief, the mesangial matrix area, which was identified by dense PAM staining and whole glomerular tuft area from each glomerulus, was measured using WinROOF image processing software (Mitani Corp., Tokyo, Japan). The mesangial matrix index was calculated using the formula [(ECM area / glomerular tuft area) x 100 (%)].

Measurement of 8-OHdG in urine

Urinary 8-OHdG concentrations after 7 months of the induction of diabetes were measured by competitive enzyme-linked immunosorbent assay kit (8-OHdG check; Japan Institute for the Control of Aging, Shizuoka, Japan) according to the manufacturer's instructions. The contents of creatinine in the same sample were determined using the Creatinine Assay Kit (Cayman, Ann Arbor, MI). Urinary 8-OHdG was expressed as ng/mg creatinine.

Immunohistochemical study

The sections were dewaxed, hydrated, and treated with 0.03 % hydrogen peroxidase for 10 min to block endogenous peroxidase activity. After blocking, the sections were reacted with either rabbit anti-human TGF-β, which is a polyclonal antibody possessing cross-reactivity with rat TGF-β (Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal antibody to pentosidine (TransGenic Inc., Kumamoto, Japan), mouse monoclonal antibody to 8-OHdG (Japan Institute for the Control of Aging, Shizuoka, Japan), or mouse monoclonal antibody to nitrotyrosine (Upstate Biotechnology Inc., Lake Placid, NY) for 1 hr at room temperature. The slides were washed with Tris buffer (pH 7.6), and then incubated with biotinylated secondary antibody for 1 hr at room temperature. The avidin-biotin-peroxidase complex method was used with a commercial kit (DAKO, Carpinteria, CA). Final development of the sections reacted with anti-8-OHdG antibody was carried out by the MaxiTags immunoperoxidase system with DAB chromogen (Shandon Lipshaw, Pittsburgh, PA, USA) and counterstained with light-green (Merck, Whitehouse Station, NJ, USA). The other sections were carried out with DAB chromogen (DAKO, Glostrup, Denmark) and counterstained with hematoxylin.

Statistical analysis

The data are expressed as mean ± SD. Comparison between groups was performed using one-way ANOVA followed by a Bonferroni / Dunn multiple comparison test. A p value less of than 0.05 was considered significant.

RESULTS

In vivo studies

Changes in body weight, plasma glucose level, and glycated hemoglobin (Hb) level during the study were presented in Fig. 1. At baseline, no significant difference was found in these three parameters among the groups. Body weight steadily increased in control rats, while diabetic rats showed almost no gain. Thus, a difference in weight between control and diabetic rats became statistically significant ($p < 0.01$) (Fig. 1a). Plasma glucose level and glycated Hb level were significantly ($p < 0.01$) higher in diabetic groups than those of control rats during the experimental period (Fig. 1b, 1c). Taurine administration did not

influence any of the parameters described above in diabetic groups. The level of BUN was increased in diabetic rats compared with control rats after 3 months of the induction of diabetes (37.2 ± 8.1 vs. 18.5 ± 3.4 mg/dl, $p < 0.01$). Taurine administration from the beginning of the fourth month showed tendency to suppress the subsequent increase in BUN in diabetic rats (control vs. untreated diabetic vs. taurine-treated diabetic; 18.8 ± 7.1 vs. 46.3 ± 3.6 vs. 38.8 ± 7.1 mg/dl at the seventh month after the induction of diabetes), though the difference in BUN level between untreated and taurine-treated diabetic rats did not reach statistical significance. On the other hand, the level of serum creatinine was significantly decreased in diabetic rats probably due to the loss of muscle tissues, so that creatinine was not a good surrogate marker to assess renal function in this animal model (control vs. untreated diabetic vs. taurine-treated diabetic; 0.39 ± 0.14 vs. 0.24 ± 0.05 vs. 0.21 ± 0.02 mg/dl at the seventh month after the induction of diabetes).
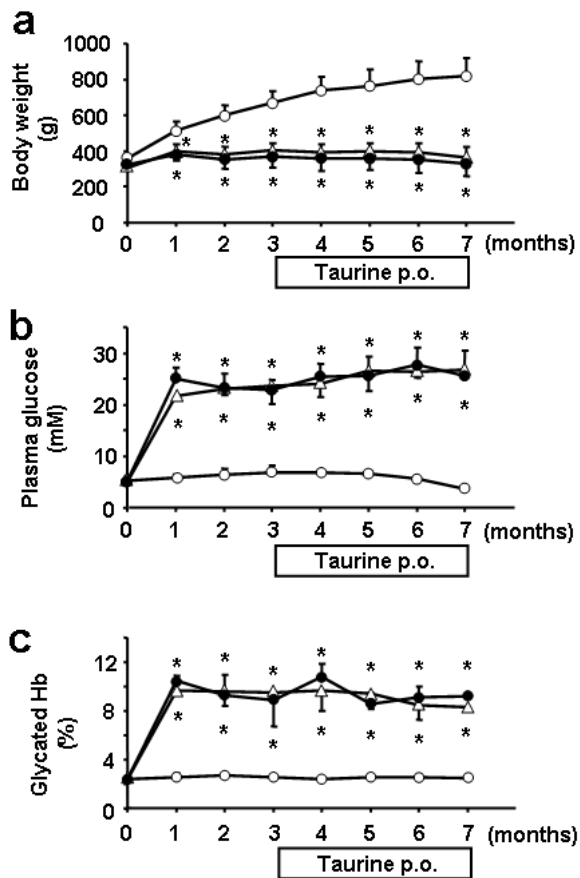

Fig. 1 Time course of changes in body weight, plasma glucose, and glycated Hb
Increase in body weight was significantly suppressed in diabetic rats (a).   Both plasma glucose (b) and glycated Hb (c) levels were significantly higher in diabetic rats compared with control rats. Treatment of diabetic rats with oral taurine did not influence any of these three parameters.
open circles = control rats; closed circles = diabetic rats; open triangles = diabetic rats with oral taurine administration starting from the beginning of the fourth month after the induction of diabetes. * p < 0.01 vs. control rats.

Urinary protein excretion rate

Urinary protein excretion rate gradually increased in diabetic rats, which became significantly different from the control group at 3 months and later (25.5 ± 6.4 vs.38.0 ± 12.2 mg/24 hr/kg BW at 3 month, $p < 0.01$, Fig. 2). Taurine administration from the beginning of the fourth month significantly suppressed the subsequent increase in urinary protein excretion in diabetic rats compared with the untreated diabetic rats (62.3 ± 49.7 vs. 114.9 ± 49.5 mg/24 hr/kg BW at 7 month, $p < 0.05$, Fig. 2).

Renal histology and morphometric analyses

Both PAS and PAM stainings showed extensive mesangial matrix expansion in the glomeruli of untreated diabetic rats compared with control rats (Fig. 3). However, taurine administration significantly suppressed the expansion of mesangial matrix in the glomeruli of diabetic rats (Fig. 3c, 3f). Quantitative analysis with the mesangial matrix index revealed that the area occupied by extracellular matrix per glomerulus in untreated diabetic rats was significantly higher than that in control rats (18.7 ± 2.3 vs. $11.0 \pm 0.3\%$, $p < 0.001$, Fig. 3g). On the other hand, the mesangial matrix index of taurine-treated diabetic rats was markedly reduced to almost control level ($12.5 \pm 2.1 \%$, $p < 0.01$ vs. untreated diabetic rats).

Urinary 8-OHdG excretion rate

Urinary 8-OHdG excretion was significantly increased in untreated diabetic rats compared with control rats (44.0 ± 40.6 vs.4.1 ± 2.2 ng/mg creatinine, $p < 0.05$, Fig. 4). Treatment of diabetic rats with taurine showed tendency to suppress the increase in excretion, but the difference did not reach statistically significant level (28.8 ± 19.2 ng/mg creatinine).

Immunohistochemical studies

Immunohistochemical studies using anti-TGF-β antibody showed intensified staining in the mesangial area of glomeruli in untreated diabetic rats at 7 months after induction of diabetes (Fig. 5b). In contrast, only a weak immunoreactivity was found in the glomeruli of age-matched control rats (Fig. 5a). Taurine administration prevented the enhancement of TGF-β expression in the glomeruli of diabetic rats (Fig. 5c). To assess the status of oxidative stress in the renal tissue, we performed immunohistochemical stainings using three different types of oxidative stress markers, such as pentosidine (Fig. 6a-c), nitrotyrosine (Fig. 6d-f), and 8-OHdG (Fig. 6g-i). As a result, intensified stainings to all these oxidative stress markers were found in the glomeruli of the untreated diabetic rats (Fig. 6b, e, h) compared with control rats (Fig. 6a, d, g). In contrast, these stainings were significantly attenuated in the taurine-treated diabetic rats (Fig. 6c, f, i). Furthermore, intensified stainings to nitrotyrosine in the vascular endothelial cells of the untreated diabetic rats were shown compared with control rats (Fig.7), which, in contrast, were also   attenuated in the taurine-treated diabetic rats (Fig. 7c).
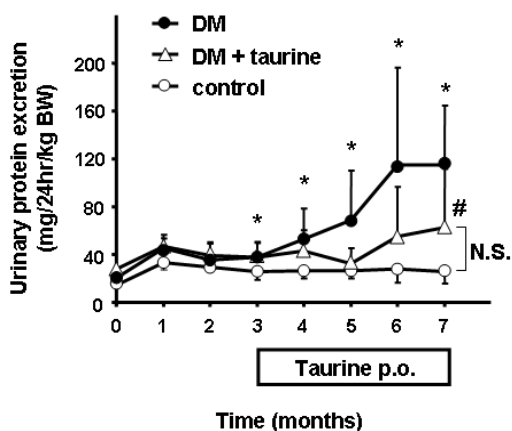

Fig. 2 Urinary protein excretion rate
Urinary protein excretion rates were significantly increased in diabetic rats (closed circles and open triangles) after 3 months of induction of diabetes compared with control rats (open circles). Treatment of diabetic rats with taurine from that time on (open triangles) significantly suppressed the increase in urinary protein excretion rates in diabetic rats.
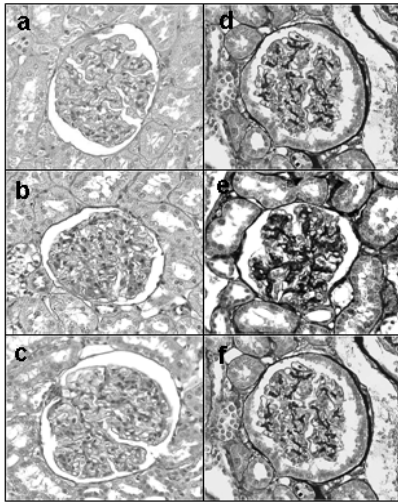* $p < 0.05$ vs. control, # $p < 0.05$ vs. untreated diabetic rats.

Fig.3 PAS and PAM stainings of renal tissues
PAS (a-c) and PAM stainings (d-f) were performed on the kidney sections. The glomeruli of diabetic rats (b, e) showed extensive matrix expansion compared with control rats (a, d). Treatment of diabetic rats with taurine considerably quenched the stainings (c, f). The mesangial matrix index of untreated diabetic rats was significantly higher than control rats, while that of taurine-treated diabetic rats was markedly reduced to almost control level(g). (x400)
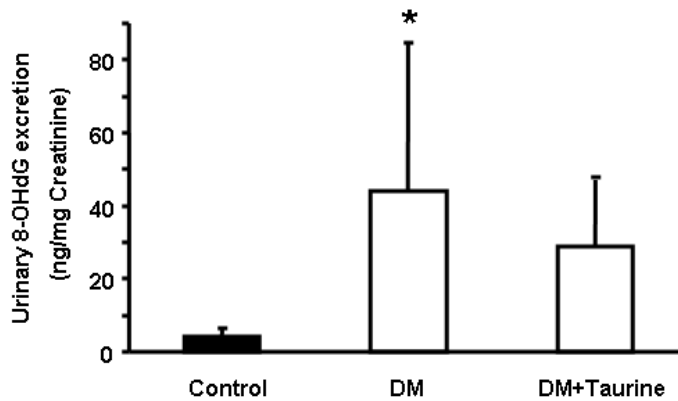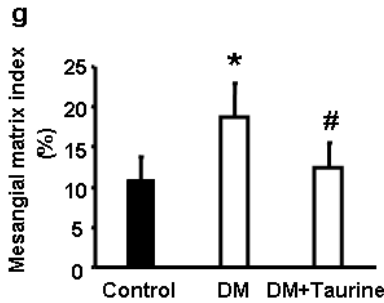* p < 0.001 vs. control, # p < 0.01 vs. untreated diabetic rats.

Fig. 4 Urinary 8-OHdG excretion rate
Urinary 8-OHdG excretion rates after 7 months of induction of diabetes in untreated diabetic rats were significantly (p < 0.05) increased compared with control rats. Treatment of diabetic rats with taurine showed tendency to suppress the increase, but the difference did not reach statistical significance.
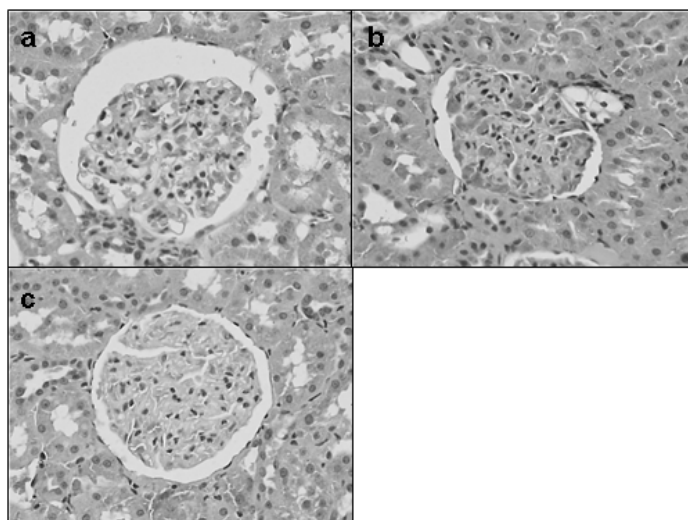* p < 0.05 vs. control rats.

Fig. 5 Immumohistochemical studies of TGF-β
Immunohistochemical staining was performed using anti-TGF-β antibody. Marked immunostaining was observed in the glomeruli of untreated diabetic rats (b) compared with control rats (a). Treatment of diabetic rats with taurine considerably quenched the immunostaining to almost control level (c). (x400)
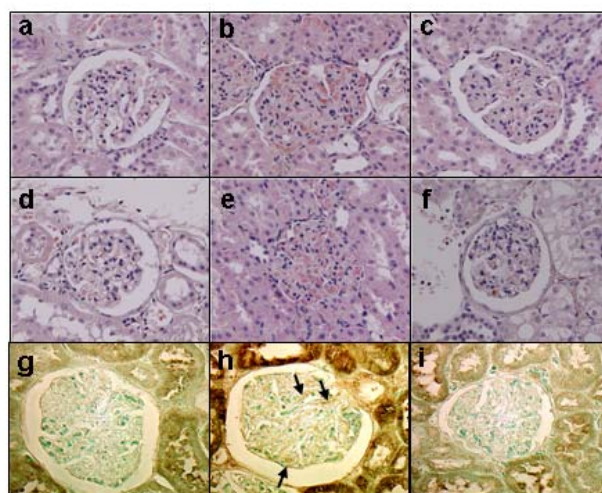

Fig. 6 Immumohistochemical studies of oxidative stress markers
Immunohistochemical stainings were performed using anti-pentosidine antibody (a-c), anti-nitrotyrosine antibody (d-f), and anti-8-OHdG antibody (g-i). Intensified immunostainings to all the markers were observed in the renal tissue of untreated diabetic rats (b, e, h) compared with control rats (a, d, g). Treatment of diabetic rats with taurine markedly quenched the enhanced immunostainings in diabetic rats (c, f, i). (x400)
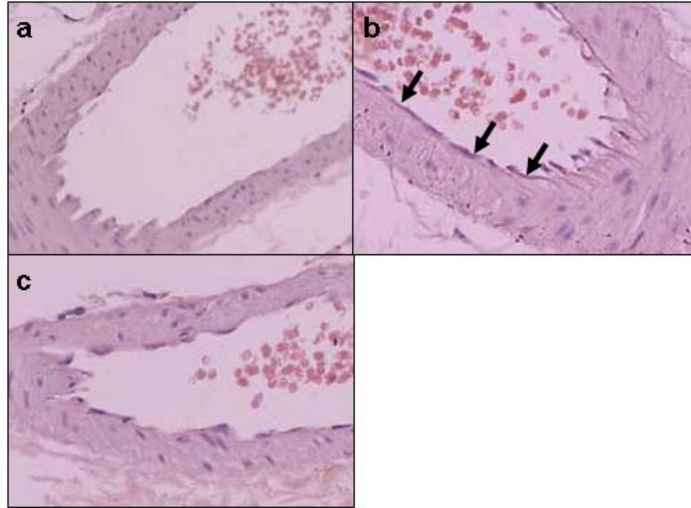
Fig. 7 Immumohistochemical detection of nitrotyrosine in renal vascular endothelial cells
Marked immunostaining (arrows) was observed in the renal vascular endothelial cells of untreated diabetic rats (b) compared with control rats (a). Treatment of diabetic rats with taurine showed tendency to attenuate the immunostaining (c). (x400)

DISCUSSION

In the present study, the development of the hallmarks of diabetic nephropathy, such as proteinuria and expansion of mesangial area, was efficiently suppressed even if taurine administration was started after the appearance of significant proteinuria. These beneficial effects of taurine were independent of blood glucose status, since both plasma glucose and glycated Hb levels were not influenced by taurine treatment. With reference to the action of taurine *in vivo*, its property as an osmolyte (18, 30), antioxidant (6, 18, 33), calcium modulator (18, 20), neurotransmitter (5), and so forth have been suggested. Among them, its antioxidant property has been paid attention in relation to animal models of kidney diseases. For example, taurine reportedly ameliorated the pathological changes in age-related progressive renal fibrosis (4) and puromycin aminonucleoside nephropathy (28) through its antioxidant property.

We also found an increased TGF-β expression in the glomeruli of STZ-induced diabetic rats, which was suppressed to almost control level in the diabetic rats with taurine treatment. TGF-β has been postulated to play an important role in the establishment of mesangial expansion by accelerating ECM production in diabetic renal tissues. Although the precise mechanism by which taurine suppressed TGF-β expression remains to be elucidated, several lines of evidence have suggested that this taurine-effect is also related to its antioxidant properties. First, a direct relationship between formation of hydrogen peroxide-induced reactive oxygen species and TGF-β expression in cultured human mesangial cells has been reported (19). Under diabetic condition, NADPH oxidase is known to play a pivotal role in the formation of reactive oxygen species. In fact, Asaba et al demonstrated that activation of NADPH oxidase in mesangial cells increased TGF-β expression and caused fibronectin accumulation in the glomerulus of diabetic rats (2). Furthermore, Studer et al. (24) showed that taurine inhibited high glucose-related overexpression of TGF-β in cultured mesangial cells, in similar fashion to that of N-acetylcystein, another antioxidant. Considering that mesangial cell is an essential cellular component in the glomerulus, these findings provide at

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least in part an explanation for taurine-induced attenuation of TGF-β expression noted in our *in vivo* study.

Thus, we next investigated the effect of taurine administration on the status of oxidative stress *in vivo*. We first evaluated urinary excretion of 8-OHdG, a marker of systemic oxidative DNA damage. As a result, taurine treatment showed a tendency to suppress the urinary excretion of 8-OHdG in diabetic rats, though the difference was not statistically significant. On the other hand, immunohistochemical study revealed that an increased intensity of 8-OHdG in the glomerulus of diabetic rats was efficiently attenuated in the taurine-treated diabetic rats. We further found that taurine treatment suppressed the intensified immunostaining to pentosidine, a glycoxidation product, which is consistent with the report by Odetti et al. (22). However, in the latter case, taurine administration was started at the induction of diabetes. In addition, nitrotyrosine, a marker of peroxynitrite-induced injury and nitrosative stress, was also attenuated by taurine treatment in the renal glomeruli of diabetic rats. Furthermore, the intensified immunostaining to nitrotyrosine was found in the renal vascular endothelial cells of untreated diabetic rats, which was attenuated by taurine treatment. In this regard, Wu et al. have demonstrated that taurine attenuates the high-glucose-induced human umbilical vein endothelial cells apoptosis partly through inhibition of intracellular reactive oxygen species formation *in vitro* (32). Therefore, the observed inhibitory effect of taurine on the formation of nitrotyrosine in the vascular endothelial cells may partly contribute to the suppression of the development of diabetic nephropathy through the prevention of hyperglycemia-induced vascular injury. These findings suggest that taurine possesses renoprotective effect mainly through local antioxidant property in diabetic animals.

Evidence for beneficial effects of taurine on diabetic nephropathy has been accumulating (13, 29). For example, Ha et al. (13) reported that taurine suppresses the expression of TGF-β at both mRNA and protein levels. Furthermore, Trachtman et al. (29) showed that taurine-administration to diabetic rats diminished immunohistochemical staining for type IV collagen in glomeruli, accompanied by a reduced malondialdehyde, a lipid peroxidation product in the kidney. However, all these results were obtained by taurine administration to animals from the time-point when they were induced diabetes. In contrast, the present study proved that the beneficial effect of taurine on diabetic nephropathy was still active even if taurine treatment was started at the time when urinary protein excretion became apparently higher than that of age-matched nondiabetic animals. Since the BUN level of diabetic rats in the present study was relatively lower than the renal failure animal models, such as lithium-induced nephropathy (7) and 5/6-nephrectomized rats (21) (BUN > 80 mg/dl), the stage of renal dysfunction in the present study seems not to the point of end-stage renal disease. Considering that pharmacological treatment usually starts after some interval from the onset of diabetes in the clinical field, we believe that the present experimental design can be modeled to actual clinical application. In addition, taurine seems quite safe in terms of adverse effects, since it is an endogenous compound. Furthermore, taurine has been reported to exhibit additional beneficial effects on diabetes-related disorders, including an increase in platelet aggregation (8, 9, 10, 11,15). The present findings, therefore, may be useful in taking another step toward prevention and treatment of diabetic nephropathy.

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