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A Possible Role of Thioredoxin Interacting Protein in the Pathogenesis of Streptozotocin-induced Diabetic Nephropathy

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Oxidative stress has been suggested to play an important role in the pathogenesis of diabetic nephropathy because it increases under diabetic conditions and is known to induce cellular dysfunction in a wide variety of cells. To protect cells against oxidative stress, cells possess defensive mechanisms such as intracellular antioxidants. Although it has been reported that central enzymes in the antioxidative defense mechanisms of the cell are induced under hyperglycemic conditions, the oxidative stress level remains high. On the other hand, there are endogenous inhibitors of antioxidants, such as thioredoxin interacting protein (Txnip). In the present study, the relationship between diabetic nephropathy and Txnip was investigated using streptozotocin (STZ)-induced diabetic mice. Eight-week-old male C57BL/6 mice were treated with either STZ or citrate vehicle. After 24 weeks of treatment, diabetic nephropathy and oxidative stress were assessed by biochemical analyses of urine and histological analyses of the kidneys. In addition, the expression of Type IV collagen α1 chain (Col4A1), Transforming growth factor-β (TGF-β), and Txnip were evaluated by real-time polymerase chain reaction. Albuminuria, renal hypertrophy, and expansion of the mesangial area, which are the hallmarks of diabetic nephropathy, were confirmed in the diabetic mice. The mRNA expression of COL4A1 and TGF-β was dramatically increased in diabetic mice in comparison with the control mice. Moreover, associated with the increased renal expression of Txnip, diabetic conditions increased oxidative stress as determined by urinary excretion of 8-hydroxy-2'-deoxyguanosine and acrolein adduct, which are oxidative stress markers. Moreover, Txnip may be a therapeutic target in diabetic nephropathy.

Diabetic nephropathy is a common cause for end-stage renal disease in industrialized countries (1,8). The characteristic features of this disease are persistent albuminuria and structural alterations, such as thickened glomerular basement membrane and progressive accumulation of extracellular matrix proteins in the glomerular mesangium (19,24). Although the number of patients with diabetic nephropathy has been increasing, the mechanisms responsible for the disease have not been clearly identified.

The involvement of various derangements associated with diabetes can be considered in the development of diabetic nephropathy. Among them, oxidative stress has been suggested to play an important role in the pathogenesis of this condition. Oxidative stress is induced by a variety of mechanisms including the glycation reaction (2), the polyol pathway (7), protein kinase C-dependent activation of membranous NADPH oxidase (9), and the mitochondrial

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electron transport chain (3,15) under diabetic conditions, and is known to induce cellular dysfunction in a wide variety of cells, such as mesangial and endothelial cells.

To protect against oxidative stress, cells possess defensive mechanisms such as intracellular antioxidants. The antioxidants regulate cellular redox balance and control the formation and reduction of reactive oxygen species (6,17,21). However, there are endogenous inhibitors of antioxidants. Thioredoxin interacting protein (Txnip) is one of such inhibitors (16) that restrains the function of thioredoxin 1, which is a major intracellular antioxidant (14). An *in vitro* study reported that high glucose levels induce Txnip expression and enhance the thioredoxin/Txnip interaction, leading to functional inhibition of antioxidative thioredoxin (23). However, the involvement of Txnip in diabetic nephropathy remains unclear. In the present study, the relationship between Txnip and diabetic nephropathy was examined using streptozotocin (STZ)-induced diabetic mice.

MATERIALS AND METHODS

Animals and experimental design

Eight-week-old male C57BL/6 mice weighing 19–26 g were randomly divided into control or diabetic groups. The number of animals per experimental group was 10. Mice in the diabetic group were intraperitoneally injected with STZ (Sigma Chemical Co., St. Louis, MO, USA) (100 mg/kg body weight in 100 µl of sterile citrate buffer, pH 4.5) for two consecutive days. The control group mice were injected with citrate vehicle. The mice with venous blood glucose levels of over 17 mmol/L were considered diabetic. The blood samples were obtained from the tail and the glucose levels were measured using Glutest-Ace (Sanwa Kagaku Kenkyusho, Nagoya, Japan). Throughout the experiment, the mice were provided with sufficient food and water. The body weight and hemoglobin A1c (HbA1c) levels were measured at 0 and 24 weeks after STZ or vehicle treatment. The venous blood HbA1c levels were determined using DCA2000 analyzer (Bayer Medical, Tokyo, Japan) from the blood sample obtained from the tail.

Sample preparation

After 24 weeks of STZ treatment, 24-h urine collection was done for each mouse using metabolic cages. The urine was kept at −20°C until urinary albumin excretion (UAE), 8-hydroxy-2'-deoxyguanosine (8-OHdG), and acrolein adduct were determined. Further, the mice were killed under ether anesthesia. The kidneys were quickly removed from the animals and the surrounding fat was cleaned. They were washed in a sterile saline solution and weighed. The right kidneys were immediately fixed by immersion in phosphate-buffered 4% paraformaldehyde solution, and routinely embedded in paraffin for light microscopy using conventional fixatives and techniques. The left kidneys were immediately frozen for gene expression analysis. The procedures were approved by the Institutional Animal Care and Use Committee guidelines at Kobe University Graduate School of Medicine.

Determination of albumin, 8-OHdG, and acrolein adduct in urine

Albuminuria was evaluated using Albuwell M (Exocell Inc., Philadelphia, PA, USA). To evaluate the degree of oxidative stress in the animals, urinary 8-OHdG, a sensitive indicator of oxidative DNA damage, and urinary acrolein adduct, a sensitive indicator of lipid peroxidation were measured. Urinary 8-OHdG and acrolein adduct levels were determined using a sandwich enzyme-linked immunosorbent assay kit (NOF Corporation, Tokyo, Japan).

Renal histology and morphometric analyses

The kidney was cut into 5-µm thick sections and stained with periodic acid Schiff (PAS) and periodic acid-silver methenamine (PAM). At least 30 glomerular tuft profiles per sample

were photographed with a digital camera (Olympus IX71; Olympus, Tokyo, Japan) and imported into Viewfinder Lite ver.1.0 (Pixera Japan, Kanagawa, Japan). The evaluation of mesangial matrix expansion was performed as described in the previous study (18). In brief, the mesangial matrix area identified by dense PAM staining and the whole glomerular tuft area from each glomerulus were measured using Lumina Vision ver.1.13 (Mitani Corporation, Fukui, Japan). The mesangial matrix index was calculated using the formula [(mesangial matrix area/glomerular tuft area) × 100 (%)]. All evaluations were performed in a blinded fashion.

Quantitative real-time PCR analysis

Type IV collagen α1 chain (Col4A1) is an extracellular matrix protein associated with the basement membrane in glomeruli (20), and Transforming growth factor-β (TGF-β) is the key regulator of extracellular matrix remodeling in the mesangium leading to mesangial expansion (26). Renal mRNA expression of these proteins and Txnip, an endogenous inhibitor of antioxidants (16), were evaluated. Total RNA was extracted from the left kidneys using RNeasy (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The RNA was dissolved in RNase-free water and stored at −80°C until it was used. Total RNA was reverse transcribed to evaluate gene expression levels. Equal amounts of total RNA from each sample were converted to cDNA by TaqMAN Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA) with random hexamer primers according to the manufacturer's manual (Applied Biosystems). Real-time quantification of the target genes was performed using a Light Cycler system (Roche Diagnostics, Basel, Switzerland). The primers were a Light Cycler primer probe set purchased from Roche Diagnostics. Relative quantities of target gene expression were compared after normalization to the value of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene.

Statistical analysis

Results were expressed as mean ± SD. Statistical analysis was performed using SPSS for Windows ver. 12.0 (SPSS Inc., Chicago, IL, USA). Data were examined by the unpaired Student's t test. $p < 0.05$ was considered statistically significant.

RESULTS

Animal profiles

The body weight and HbA1c levels at the beginning and end of the study are shown in Table 1. It was observed that the body weight increased in the control mice. In contrast, diabetic mice showed little gain in weight. Therefore, the body weights of the diabetic animals were significantly ($p < 0.01$) reduced in comparison with the controls at the end of the experiment. HbA1c levels in the controls did not change throughout the experiment. In contrast, HbA1c levels were significantly ($p < 0.01$) higher in the diabetic group than in the control group. These results indicate that hyperglycemia in diabetic mice was persistent and not transient.

Urinary albumin excretion and renal hypertrophy

An increase in the levels of UAE and renal hypertrophy are the earliest manifestations of renal disorder in diabetes. The 24-h UAE was markedly ($p < 0.01$) higher in diabetic mice than in control mice (Fig. 1A). Furthermore, the kidney weight/body weight ratio was significantly ($p < 0.01$) increased in diabetic mice in comparison with the control mice in the 24th week after STZ treatment (Fig. 1B). These results indicate that the renal disorder in STZ-induced diabetic mice, which is early stage diabetic nephropathy, is similar to that seen in humans.

TABLE 1. Changes in body weight and HbA1c levels.

	Body weight (g)		HbA1c levels (%)	
	Initial	Final	Initial	Final
Control mice	22.65 ± 1.44	31.77 ± 2.94	3.0 ± 0.3	3.4 ± 0.4
Diabetic mice	22.29 ± 1.58	24.10 ± 2.85*	2.9 ± 0.4	10.0 ± 1.8*

Data were obtained at 0 and 24 weeks after STZ or citrate vehicle treatment. The body weights in the diabetic animals were significantly reduced in comparison with the control values at the end of the experiment. HbA1c levels were significantly higher in diabetic groups than in the control groups.
Data are presented as means ± SD. *$p < 0.01$ vs. control mice.

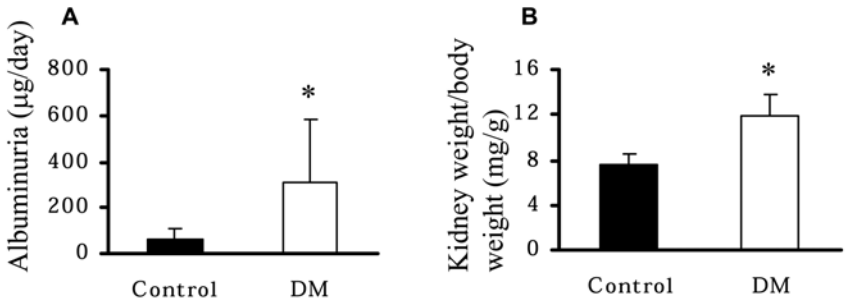

FIG. 1. Albuminuria and renal hypertrophy
The 24-h urinary albumin excretion was markedly higher in diabetic mice than in control mice (A). Furthermore, the kidney weight/body weight ratio was significantly increased in diabetic mice in comparison with the control mice at the 24th week after STZ treatment (B). Data are mean ± SD. Closed bar = control mice; open bar = diabetic mice. *$p < 0.01$ vs. control mice.

Morphologic changes of the kidney

The diabetic mice showed extensive mesangial matrix expansion (Fig. 2B, D), whereas no change was observed in the control mice (Fig. 2A, C). Quantitative analysis of the mesangial matrix index of at least 30 glomeruli per mouse revealed that the area occupied by matrix per glomerulus was significantly ($p < 0.01$) greater in diabetic mice than in control mice (Fig. 2E).

Col4A1 and TGF-β mRNA expression in renal tissue

The mRNA expression of COL4A1, which is an extracellular matrix protein that is associated with the basement membrane in glomeruli, was dramatically ($p < 0.01$) increased in diabetic mice in comparison with the control mice (Fig. 3A). In addition, the mRNA expression of TGF-β, which is the key regulator of extracellular matrix remodeling in the mesangium leading to mesangial expansion, was significantly ($p < 0.01$) increased in the diabetic animals (Fig. 3B).

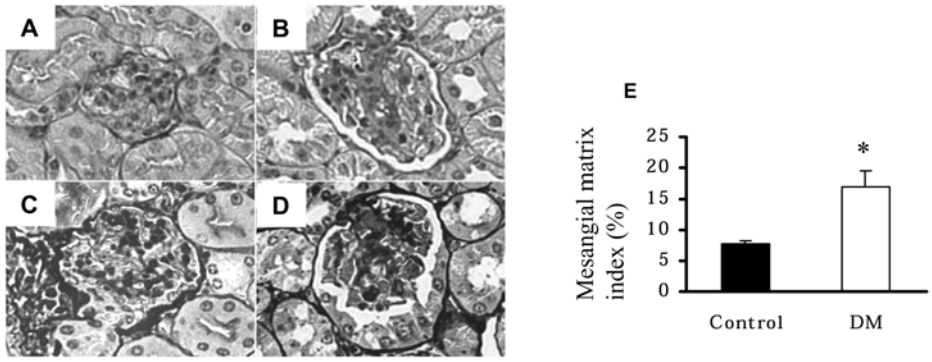

FIG. 2. Mesangial matrix expansion
The kidneys were removed after 24-weeks of STZ or vehicle treatment. Renal tissues were stained with PAS (A, B) and PAM (C, D). Diabetic mice showed extensive mesangial matrix expansion (B, D), whereas no change was observed in control mice (A, C). Quantitative analysis revealed that diabetic mice demonstrated a significant increase in the area occupied by matrix per glomerulus in comparison with the control mice (E). Magnification is × 400. Data are mean ± SD. Closed bar = control mice; open bar = diabetic mice. *$p < 0.01$ vs. control mice.

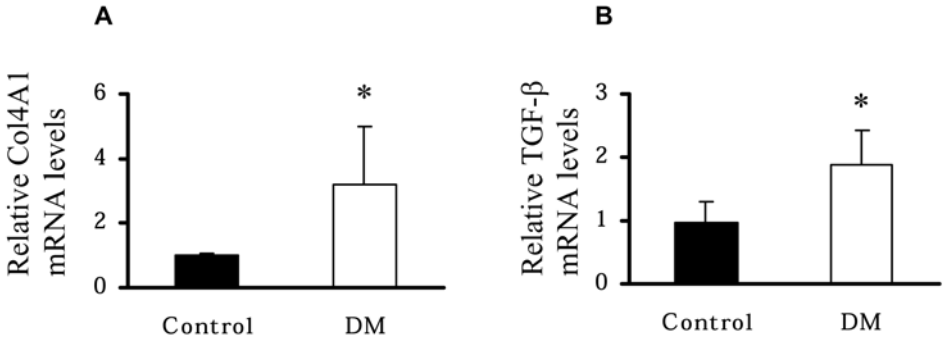

FIG. 3. The expression of Col4A1 and TGF-β mRNA in renal tissue
Gene expression was analyzed using quantitative real-time PCR by comparing the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. (A) The expression of Col4A1 mRNA. (B) The expression of TGF-β mRNA. Data are mean ± SD. Closed bar = control mice; open bar = diabetic mice. *$p < 0.01$ vs. control mice.

Oxidative stress makers and Txnip mRNA expression

To evaluate the degree of oxidative stress in the mice, the urine levels of 8-OHdG and acrolein adduct were determined. Both markers were remarkably ($p < 0.01$) higher in diabetic mice than in the control mice (Fig. 4A, B). Furthermore, quantitative real-time PCR analysis revealed that the mRNA expression of Txnip in the kidney was significantly ($p < 0.01$) increased in diabetic mice in comparison with the control mice 24 weeks after STZ treatment (Fig. 4C).

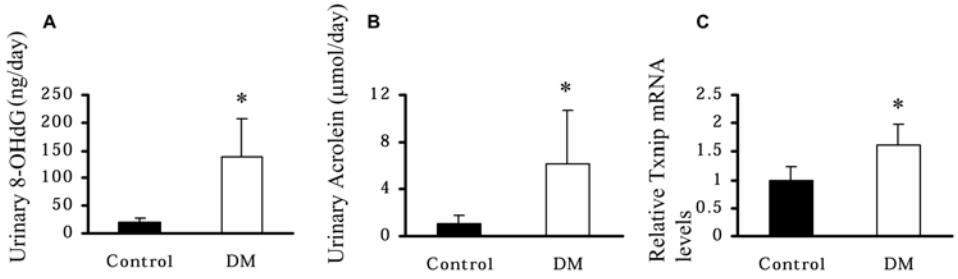

FIG. 4. Oxidative stress makers and Txnip mRNA expression
The excretion of urinary 8-OHdG (A) and acrolein adduct (B) were remarkably higher in diabetic mice than the control mice. Quantitative real-time PCR analysis revealed that the mRNA expression of Txnip in the kidney was significantly increased in diabetic mice in comparison with the control mice at 24 weeks after STZ treatment (C). Data are mean ± SD. Closed bar = control mice; open bar = diabetic mice. *$p < 0.01$ vs. control mice.

DISCUSSION

In the present study, albuminuria, renal hypertrophy, and expansion of mesangial area, which are the hallmarks of diabetic nephropathy, were confirmed in STZ-induced diabetic mice. In addition, the mRNA expression of COL4A1 and TGF-β was dramatically increased in diabetic mice in comparison with the control mice. Collagen accumulation is the hallmark of glomerular sclerosis, and increase of COL4A1 plays a central role in this process (22). Moreover, TGF-β is the key regulator of extracellular matrix remodeling in the mesangium leading to mesangial expansion (26), and induction of TGF-β is a well-documented molecular event during the development of diabetic nephropathy both *in vitro* (10,27) and *in vivo* (10,25). Taken together, these results suggest that the nephropathy in the STZ-induced diabetic mice mimics the diabetic nephropathy seen in humans with diabetes.

Among the various derangements associated with diabetes, oxidative stress has been suggested to play an important role in the pathogenesis of diabetic nephropathy. Our results demonstrate that diabetic conditions increase oxidative stress as determined by urinary excretion of 8-OHdG (a sensitive indicator of oxidative DNA damage) and acrolein adduct (a sensitive indicator of lipid peroxidation). These findings suggest a relationship between oxidative stress and the development of diabetic nephropathy.

To protect against increased oxidative stress under diabetic conditions, it is reported that superoxide dismutase, glutathione peroxidase, and catalase, which are central enzymes in the antioxidative defense mechanisms of cells, are induced under hyperglycemic conditions (4). Moreover, it has been reported that thioredoxin 1, which is a major intracellular antioxidant (14), is induced under diabetic conditions (11,12). TRX is cytokine-like factor with radical-scavenging functions (5,13,14), and it has been suggested that the regulation of cellular reduction/oxidation (redox) by TRX plays an important role in signal transduction and cytoprotection against oxidative stress (5,13,14). However, oxidative stress levels remain high in the diabetic milieu.

In the present study, we demonstrated that renal expression of Txnip increased dramatically in diabetic mice. Since Txnip inhibits the antioxidative function of thioredoxin (16), our results suggest that diabetic conditions enhance the thioredoxin/Txnip interaction, leading to functional inhibition of the antioxidative thioredoxin function. The interaction results in a shift of the cellular redox balance that promotes increased intracellular oxidative stress. In other words, an increase in Txnip expression may be a mechanism by which

oxidative stress levels remain high under diabetic conditions. To clarify the mechanism of the development of diabetic nephropathy, further studies linking diabetic nephropathy to oxidative stress and Txnip are required.

In conclusion, our study demonstrated increased oxidative stress associated with Txnip induction in STZ-induced diabetic nephropathy. Moreover, Txnip may be a therapeutic target in diabetic nephropathy.

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