Overexpression of upstream stimulatory factor 2 accelerates diabetic kidney injury

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Liu S, Shi L, Wang S. Overexpression of upstream stimulatory factor 2 accelerates diabetic kidney injury. Am J Physiol Renal Physiol 293: F1727–F1735, 2007. First published September 19, 2007; doi:10.1152/ajprenal.00316.2007.—Diabetic nephropathy is the most common cause of end-stage renal failure in the United States. Hyperglycemia is an important factor in the pathogenesis of diabetic nephropathy (3, 20, 27). Moreover, studies have shown that anti-TGF-β antibodies prevent the development of diabetic nephropathy (2, 32), providing a direct evidence for the involvement of TGF-β in the pathogenesis of diabetic nephropathy. The fibrogenic effects of TGF-β are due to its upregulation of extracellular matrix components, including collagen, fibronectin, osteopontin, and the downregulation of matrix-degrading enzymes (12, 40).

Studies demonstrated that the matricellular protein, thrombospondin 1 (TSP1), is a major regulator of TGF-β activation and extracellular matrix protein expression by renal mesangial cells under high-glucose conditions (21, 35, 38). However, the mechanisms by which high glucose upregulates TSP1-dependent TGF-β activity are not well-understood. Previously, we demonstrated that the transcription factor, upstream stimulatory factor 2 (USF2), was upregulated by high glucose, which bound to an 18-bp sequence in the thrombospondin 1 (TSP1) gene promoter and regulated high-glucose-induced TSP1 expression and TGF-β activity in mesangial cells, suggesting that USF2 might play a role in the development of diabetic nephropathy. In the present studies, we examined the effect of overexpression of USF2 on the development of diabetic nephropathy. Type 1 diabetes was induced in USF2 transgenic mice [USF2 (Tg)] and their wild-type littermates (WT) by injection of streptozotocin. Four groups of mice were studied: control WT, control USF2 (Tg), diabetic WT, and diabetic USF2 (Tg). Mice were killed after 15 wk of diabetes onset. At the end of studies, control USF2 (Tg) mice (~6 mo old) exhibited increased urinary albumin excretion. These mice also exhibited glomerular hypertrophy, accompanied by increased TSP1, active TGF-β, fibronectin accumulation in the glomeruli compared with control WT littermates. Type 1 diabetes onset further augmented the urinary albumin excretion and glomerular hypertrophy in the USF2 (Tg) mice. These findings suggest that overexpression of USF2 accelerates the development of diabetic nephropathy.
OVEREXPRESSION OF USF2 ACCELERATES DIABETIC NEPHROPATHY

Fig. 1. Generation of transgenic mice with overexpression of mouse upstream stimulatory factor 2 (USF2) protein. A: schematic depiction of the mouse USF2 transgene fragment used to generate USF2 transgenic mice. B: representative RT-PCR results showing the transgene overexpressed in different tissues from transgenic mice.

Characterization of Transgenic Mice

Reverse transcriptase-PCR to determine the expression levels of transgene in different tissues. Total RNA was extracted from the kidneys of USF2 transgenic (Tg) mice and their WT littermates using TRIzol (Invitrogen, Carlsbad, CA). Total RNA (2 μg) was reverse transcribed in a 20-μl reaction using a random hexamer primer and Thermoscript RT (Invitrogen) at 55°C for 50 min. Of this cDNA, 2 μl were added to the PCR. Each PCR was conducted in a total volume of 25 μl with Platinum Taq DNA polymerase. The conditions for PCR were 95°C for 10 min followed by 35 cycles of PCR amplification with the denaturing at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. β-Actin was used as a control to monitor RT-PCR amplification. PCR products were separated by electrophoresis in a 1.5% agarose gel and visualized by ethidium bromide staining and UV illumination. The primers used for PCR of USF2 (295 bp) were 5′-TGGCATAGAGGTCTTTACGG-3′ and 5′-ACAGACCAGAAGCCTACAGGC-3′, and β-actin (474 bp) were 5′-CACGCGATTTG-GATGAGCT-3′ and 5′-TGCCAATAGGTTCTTTAACGG-3′. The PCR products were digested with Sal I, SnaBI, EcoR I, and Bgl II. Right kidneys were fixed with 4% paraformaldehyde. Total RNA was extracted from kidney using TRIzol (Invitrogen) and Northern blotting was performed to examine the overexpressed mRNA levels of USF2 in transgenic mice. For Northern blot analysis, equal amounts of total RNA (10 μg) were denatured, electrophoresed, and transferred to nylon membranes. RNA was fixed by UV cross-linking. The USF2 probe (mouse USF2 cDNA, generously provided by Dr. Sawadogo at the University of Texas MD Anderson Cancer Center) was radiolabeled by random prime DNA-labeling kit (Roche Molecular Biochemicals) with [α-32P]dCTP. The membrane was prehybridized in ExpressHyb hybridization solution (Clontech, Palo Alto, CA) for 30 min at 68°C, and hybridization was carried out at 68°C for 1 h in fresh hybridization solution with denatured probe. The membrane was washed three times with 2× SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate), 0.05% SDS for 15 min at room temperature, and then twice with 0.2× SSC, 0.1% SDS at 50°C for 20 min. Blots were developed, and films were scanned. The absorbance units were corrected for the β-actin levels. In addition, USF2 protein levels in kidney homogenates were determined by Western blotting as described previously (36). Finally, fixed kidneys were embedded in paraffin, and 5-μm sections were stained with anti-USF2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) using Vectastain Elite ABC kit (Vector).

Experimental Animals and Protocol

Eight-week-old male USF2 (Tg) mice and sex- and age-matched WT littermates were used in the studies. All these mice were on B6C3H background and were cared for in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals. Diabetes was induced in the mice at the age of 8 wk by injecting streptozotocin (STZ; 40 mg/kg) intraperitoneally for 5 consecutive days. STZ (Sigma, St. Louis, MO) was prepared in 0.1 mol/l sodium citrate buffer (pH 4.5) immediately before injection. Control animals received injections of citrate buffer alone. The development of diabetes was evaluated by measuring blood glucose using Glucometer 2 wk after the first injection, and the mice with a blood glucose level higher than 300 mg/dl were considered diabetic and were used for the study. There were four groups: 1) control WT, 2) control USF2 (Tg); 3) diabetic WT; and 4) diabetic USF2 (Tg). Each group contained 10 mice. These mice were kept for 15 wk. Throughout the studies, mice were provided with free access to water and normal standard mouse chow. Blood glucose levels and body weights were measured biweekly. At the end of the study, 24-h urine was collected. Mice were killed at 15 wk after diabetes onset. All protocols were approved by the institutional animal care and use committee of University of Kentucky. All experiments were conducted in accordance with the guidelines of the University of Kentucky Animal Care Committee.

Measurement of Urinary Albumin Excretion

At 15 wk, 24-h urine was collected on individual mice housed in metabolic cages with free access to chow and water. Total urine volume was measured. Urinary albumin was determined using the Albuvell M Murine Microalbuminuria enzyme-linked immunosorbent assay kit (Exocell, Philadelphia, PA). Results were reported as micrograms of albumin per 24-h urine. Urinary creatinine concentration was measured using a picric acid assay (Exocell).

Immunoblot Analysis

Glomerular expression of USF2, fibronectin, TSP1, and active TGF-β was assessed by Western blot analysis. Mice in each group (n = 3 per group) were killed and kidneys were collected. The glomeruli were isolated by differential sieving (150-, 106-, 90-, and 53-μm sieves) (24) and lysed in Laemmli SDS sample buffer, and 30 μg of protein were subjected to SDS-PAGE on 12% gel under reducing condition and transferred onto PVDF membrane. After being blocked, the membrane was incubated with polyclonal anti-USF2 antibody (Santa Cruz Biotechnology), monoclonal anti-TSP1 antibody (A4.1, GIBCO-BRL), monoclonal anti-TGF-β1, 2, 3 antibody (R&D System), or rabbit anti-rat fibronectin antibody (from Life
Technology) for 1 h at room temperature. After being washed, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody (Jackson Laboratory). The reaction was visualized using an enhanced chemiluminescence system (Pierce).

EMSA

Mice were killed, and the kidneys were harvested. The glomeruli were isolated by a fractional sieving technique. Glomeruli were washed with cold PBS, and nuclear extracts were prepared as described previously (26, 36) and used for the EMSA assay. The 55-bp oligonucleotide of TSP1 promoter (−932 to −878) (36) was end-labeled with [32P] by T4 polynucleotide kinase (Invitrogen). A probe (2–5 × 10⁶ cpm) was incubated with 3 μg of nuclear extracts in a 20-μL volume of binding reaction buffer [10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10% glycerol, 50 ng/ml poly(dI/dC)] on ice for 20 min. In competition experiments, an unlabeled USF2 consensus oligonucleotide (Santa Cruz Biotechnology) was mixed with the labeled probe before being added to the binding mixture to determine the specificity of nuclear binding. The binding reaction was then allowed to proceed for 20 min on ice. All binding mixtures were separated by 4–20% gradient Tris-buffered TBE gels (Bio-Rad). The gels were dried and exposed to film.

Real-time PCR

Total RNA (2 μg) extracted from glomeruli of control and diabetic mice was converted to cDNA using MLV reverse transcriptase (Promega). The validity of primers and appropriate melting temperature for real-time PCR were determined to ensure that only one band is amplified by PCR reaction. Primers were synthesized by Integrated DNA Technologies, with sequences as follows: 1) mouse fibronectin (399 bp): forward 5′-AGCAGTGGAACGCCAAGCT-3′ and reverse 5′-ACGTAGGAGTCACAGCAGC-3′, 2) mouse TSP1 (361 bp) forward 5′-GGAGCCGCTGGATCTCTGGA and reverse 5′-CTTCTTGGAAATGCGGCAA-3′, and 3) mouse TGF-β1 (430 bp): forward 5′-CTAATGGTGACGCAGCACAAC-3′ and reverse 5′-CGGTTCATGTCATGGATGGTG-3′. Real-time PCR analyses were performed using SYBR Green PCR Master Mix kit with a MiYQ real-time PCR thermal cycler (Bio-Rad). For each target gene, a standard curve was established by a series of threefold dilution of the gene of interest. PCR conditions. Mesangium was defined by the PAS-positive staining by semiquantitative method by a reader blinded to the experimental conditions. Mesangial expansion was end-accepted at a value of P < 0.05.

RESULTS

Characterization of the USF2 Tg Mice

The USF2 transgenic mice were viable and fertile. There were no obvious physical abnormalities observed in these mice. Six-week-old male mice were used for characterization. RT-PCR results showed that transgene overexpressed in various tissues including heart, muscle, liver, lung, kidney, aorta, spleen, testes, brain, intestine, etc (Fig. 1B). To further analyze the expression of transgene in the kidney, we performed Northern blotting, Western blotting, and immunohistochemical staining. As shown in Fig. 2, in the kidney of transgenic-positive mice, USF2 mRNA levels were 5-fold and protein levels were 3.5-fold above the level in control mice. In addition, a significant increase in positive staining of USF2 was shown in both glomeruli and tubular system in the USF2 transgenic mice (Fig. 2C). Taken together, these data indicate that overexpression of USF2 occurred in the kidneys as well as other tissues in the USF2 transgenic mice.

General Characteristics in Control and Diabetic Mice

To direct determine the effect of overexpression of USF2 on the development of diabetic nephropathy, USF2 transgenic mice and their WT littermates were made diabetic by STZ injection as described in MATERIALS AND METHODS. General characteristics of control and diabetic mice at week 15 including body weight, blood glucose levels, kidney weight, urine volume, and 24-h urine albumin excretion were shown in Table 1. Reduction in body weight was observed in diabetic WT mice and USF2 (Tg) mice. Blood glucose levels were elevated 2 wk after the first STZ injection in both diabetic WT and diabetic
USF2 (Tg) groups of mice compared with their nondiabetic controls, and remained elevated throughout the study period. At 15 wk, there was no difference between the blood glucose levels in diabetic USF2 (Tg) mice and diabetic WT mice.

At the end of the studies, the ratio of kidney weights to body weights was significantly increased in nondiabetic USF2 (Tg) mice (~6 mo old) compared with nondiabetic WT group. The diabetes onset significantly augmented the ratio of kidney weights to body weights in USF2 (Tg) mice (Table 1). The 24-h urine volume and urinary albumin excretion were significantly greater in nondiabetic USF2 (Tg) group compared with nondiabetic WT group. Under diabetes conditions, 24-h urine volume and urinary albumin excretion were increased in both USF2 (Tg) and WT groups compared with their nondiabetic controls. There was a significant increase in 24-h urine volume and urinary albumin excretion in diabetic USF2 (Tg) mice compared with diabetic WT group. These studies demonstrated that USF2 (Tg) mice exhibited albuminuria at 6 mo of age, which were significantly augmented by diabetes onset.

Renal Histology Analysis in Control and Diabetic Mice

Given the marked effect of overexpression of USF2 gene on the urinary albumin excretion, we further determined whether there were differences in the kidney structure (Fig. 3, A and B). The PAS staining was performed. Glomerular size was determined to assess glomerular hypertrophy. The glomerular size was significantly increased in diabetic WT (D-WT vs. WT, \(3.7 \times 10^3 \pm 68\) vs. \(2.5 \times 10^3 \pm 89, P < 0.05\) or diabetic USF (Tg) mice [D-USF (Tg) vs. USF2 (Tg), \(5.08 \times 10^3 \pm 102\) vs. \(3.3 \times 10^3 \pm 76, P < 0.05\) compared with their control mice. We also determined the mesangial matrix expansion. The mean values for the mesangial matrix scores were shown in Fig. 3B. Mean values for mesangial matrix score were significantly increased in both diabetic groups compared with their nondiabetic controls. The diabetic USF2 (Tg) mice developed a greater increase in mesangial matrix score than that in diabetic WT mice.

Expression of USF2 in Glomeruli From Control and Diabetic Mice

We showed that high-glucose levels upregulated USF2 expression in mesangial cells in vitro (36), and to determine whether diabetes upregulated renal USF2 expression from diabetic mice, glomeruli were isolated and immunoblotting was performed (Fig. 4A). As expected, glomeruli USF2 protein levels were increased in diabetic WT mice compared with the nondiabetic WT control. In USF2 (Tg) mice, diabetes onset further increased glomeruli USF2 levels. These data provided...
the first evidence that type 1 diabetes onset stimulated USF2 expression in glomeruli.

Binding of USF2 to a 55-bp TSP1 Promoter Region (−932 to −878) by EMSA

Previously, we demonstrated that high glucose upregulated USF2 expression in mesangial cells and stimulated USF2 to bind to a 55-bp TSP1 promoter region (−932 to −878) (36). In this study, we determined whether nuclear extracts of glomeruli from USF2 (Tg) mice showed increased binding to the TSP1 promoter by using EMSA. As shown in Fig. 4B, a major band formed in EMSA. This band was significantly enhanced in both of the diabetic groups compared with their nondiabetic controls. There was also a marked increase in protein-DNA interaction in the diabetic USF2 (Tg) mice compared with the diabetic WT mice. Competition studies showed that unlabeled USF2 consensus oligonucleotide efficiently abrogated the formation of this complex (Fig. 4B, lane 1). Together, these data demonstrated that glomerular USF2 binding to the TSP1 promoter was increased under diabetes conditions.

Renal Expression of Fibronectin in Control and Diabetic Mice

To characterize further the mesangial injury, immunohistochemical staining, real-time PCR, and Western blotting were used to evaluate the expression of the extracellular matrix protein fibronectin in the kidney from four groups of mice (Fig. 5). There was a twofold increase in glomerular fibronectin immunostaining in the diabetic WT mice compared with nondiabetic WT controls and a threefold increase in glomerular fibronectin immunostaining in the diabetic USF2 (Tg) mice compared with nondiabetic USF2 (Tg) mice. Fibronectin immunostaining was greater in diabetic USF2 (Tg) mice compared with diabetic WT mice. Similarly, the mRNA (Fig. 5B) and protein levels of fibronectin (Fig. 5C) in isolated glomeruli were significantly increased in both diabetic mice compared with their control mice. The levels of fibronectin (mRNA and protein) were greater in diabetic USF2 (Tg) compared with diabetic WT mice.

Renal Expression of TSP1 and Active TGF-β in Control and Diabetic Mice

Our previous studies demonstrated that USF2 binds to TSP1 gene promoter and regulates high glucose-mediated TSP1 expression and TGF-β activity in glomerular mesangial cells (36). To determine whether overexpression of USF2 stimulates TSP1 expression and increase in active TGF-β levels in the
kidney from USF2 (Tg) mice under basal and diabetic conditions, we performed real-time PCR, Western blotting, and immunohistochemical staining for TSP1 and active TGF-β (Figs. 6 and 7). TSP1 and active TGF-β immunostaining and mRNA and protein levels of TSP1 and active TGF-β were increased in both of the diabetic groups compared with their nondiabetic controls. There was also a marked increase in TSP1 and active TGF-β levels in the diabetic USF2 (Tg) mice compared with the diabetic WT mice.

DISCUSSION

USF2 was initially characterized as a transcription factor implicated in the regulation of the adenovirus major late gene.
and induced TSP1 gene expression and TGF-USF2 accumulation, which mediated high-glucose conditions demonstrated that high-glucose exposure stimulated nuclear 10, 13, 17, 22, 23, 34, 39). In our previous studies, we been demonstrated to regulate expression of many genes (4, 9, (31). Through binding to E boxes of target genes, USF2 has recognize in vitro a CACGTG core sequence termed E box and DNA binding. It can form homo- and heterodimers and loop helix/leucine zipper domain responsible for dimerization family of transcription factors characterized by a basic/helix with a molecular weight of 44 kDa. It belongs to the Myc promoter (25). In mammals, USF2 is ubiquitously expressed in glandular mesangial cells, suggesting a role of USF2 in the development of diabetic renal complications. The current studies assessed the role of USF2 in short-term diabetes focusing on the effect of overexpression of USF2 on diabetic renal injury. The findings of the present study provide the first evidence that overexpression of USF2 accelerates the development of diabetic nephropathy.

It is known that one of the earliest clinically detectable functional abnormalities in diabetic nephropathy is microalbuminuria (37). Accordingly, we first determined whether overexpression of USF2 accelerates the development of diabetic nephropathy. The degree of renal functional changes (albuminuria) correlates with the progression of renal structural changes including mesangial matrix expansion, glomerulosclerosis, and tubulointerstitial fibrosis (16). Therefore, next, we determined whether the exacerbation of urinary albumin excretion in diabetic USF2 (Tg) mice relates to structural changes in the kidneys. We found that the ratio of left kidney to body weight was greater in diabetic USF2 (Tg) mice than that in diabetic WT mice. We performed PAS staining to determine the mesangial matrix expansion in four groups of animals. We also determined the glomerular size to assess glomerular hypertrophy. The mean values of mesangial matrix scores significantly increased in USF2 (Tg) mice under both nondiabetic and diabetic conditions compared with control WT mice. Diabetic USF2 (Tg) mice exhibited a greater mean value for mesangial matrix scores than diabetic WT littermates. Glomerulosclerosis and tubulointerstitial fibrosis were not observed in any group of mice, suggesting that the kidney injuries observed in the diabetic mice are still early features of diabetic nephropathy. In addition, immunohistochemical studies, real-time PCR, and Western blotting analysis of the kidneys demonstrated that diabetic USF2 (Tg) mice and diabetic WT littermates exhibited greater fibronectin immunostaining, mRNA, and protein levels of fibronectin in glomeruli compared with their nondiabetic controls. Fibronectin expression was most pronounced in the diabetic USF2 (Tg) mice, contributing to the increased mesangial matrix expansion in these mice. These observations indicated that USF2 (Tg) mice at 6 mo of age developed glomerular hypertrophy and mesangial matrix expansion, which was augmented by diabetes onset.

We further determined the mechanisms for the acceleration of the functional and structural changes of diabetic nephropathy in USF2 (Tg) mice. Accumulating evidence suggests that TGF-β plays a key role in the development of diabetic nephropathy by upregulation of extracellular matrix components, including collagen, fibronectin, osteopontin, and the down-regulation of matrix-degrading enzymes (12, 40). We and
others demonstrated that the matricellular protein, TSP1, is a major regulator of TGF-β activation and extracellular matrix protein expression by renal mesangial cells under high-glucose conditions (21, 35, 38). Our studies also demonstrated that USF2 regulated high glucose-induced TSP1 expression and TGF-β activity in mesangial cells (36). In the current studies, we provided first evidence that hyperglycemia stimulated glomerular USF2 expression from diabetic WT control mice and USF2 (Tg) mice and enhanced USF2 binding to the TSP1 promoter. TSP1 and active TGF-β levels (mRNA and protein) in glomeruli were significantly increased in nondiabetic USF2 (Tg) mice, diabetic WT littermates, and diabetic USF2 (Tg) mice. Active TGF-β levels were most pronounced in the diabetic USF2 (Tg) mice, supporting our conclusion that differences in active TGF-β levels were responsible for the accelerated diabetic kidney injury in the USF2 (Tg) mice.

In addition to the importance of TGF-β in the development of diabetic kidney injury in USF2 (Tg) mice, other factors may need to be explored. It has been shown that USF2 regulates renin gene expression (19). Multiple evidence showed that the activation of intrarenal renin-angiotensin system (RAS) plays an important role in diabetic nephropathy. Blockade of RAS by angiotensin-converting enzyme inhibitors and angiotensin II receptor antagonists has been shown to attenuate proteinuria and slows the progression of diabetic nephropathy (1, 6–8, 14, 15). However, whether USF2 overexpression in the kidney upregulates intrarenal RAS and contributes to the development of nephropathy in USF2 (Tg) mice under both nondiabetic and diabetic conditions is not known. Given the importance of intrarenal RAS in diabetic nephropathy, the effect of USF2-mediated renin expression on the development of diabetic nephropathy will be investigated.

In summary, we provided the first evidence that hyperglycemia stimulated USF2 expression in glomeruli from diabetic control mice, which was associated with increased TSP1 expression and active TGF-β levels, and fibronectin accumulation in glomeruli, leading to the development of diabetic nephropathy. In addition, overexpression of USF2 in the kidney further augmented the above features and exacerbated the diabetic kidney injury.

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GRANTS

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