High Glucose Upregulates Upstream Stimulatory Factor 2 in Human Renal Proximal Tubular Cells through Angiotensin II-Dependent Activation of CREB

Nishant P. Visavadiya Yanzhang Li Shuxia Wang

Graduate Center for Nutritional Sciences, University of Kentucky, Lexington, Ky., USA

Key Words

Upstream stimulatory factor 2 • High glucose • Renin-angiotensin system • Renal proximal tubular cells • cAMP response element-binding protein (CREB) • CREB

Abstract

Background/Aims: We have previously demonstrated that a transcription factor, upstream stimulatory factor 2 (USF2), regulates glucose-induced thrombospondin 1 expression and transforming growth factor- β activity in mesangial cells, and plays an important role in diabetic glomerulopathy. In this study, we determined whether USF2 expression in renal proximal tubular cells is regulated by glucose and contributes to diabetic tubulointerstitial fibrosis. **Methods:** Human renal proximal tubular cells (HK-2 cells) were treated with normal- or high-glucose medium for 24 h. After treatment, real-time PCR or immunoblotting was used to determine the expression of USF2 and other components of the renin-angiotensin system in HK-2 cells. **Results:** High glucose upregulated USF2 expression and increased extracellular matrix accumulation in HK-2 cells; both were inhibited by siRNA-mediated USF2 knockdown. In addition, high glucose stimulated angiotensinogen and renin expression, increased renin activity, and resulted in increased angiotensin II formation. Treatment of HK-2 cells with an angiotensin II receptor 1 (AT1)

blocker – losartan – prevented high-glucose-induced USF2 expression and high-glucose-enhanced phosphorylation of CREB (cAMP response element-binding protein). **Conclusion:** Our data established that high glucose stimulated USF2 expression in HK-2 cells, at least in part, through angiotensin II-AT1-dependent activation of CREB, which can contribute to diabetic tubulointerstitial fibrosis.

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Introduction

Diabetic nephropathy (DN) is the most common cause of end-stage renal failure. About 20–30% of people with type 1 and type 2 diabetes develop DN. DN is characterized by both glomerulosclerosis with thickening of the glomerular basement membrane and mesangial matrix expansion, and tubulointerstitial fibrosis [1]. The renal proximal tubular cells respond to hyperglycemia and produce excessive amounts of extracellular matrix proteins, contributing to diabetic tubulointerstitial fibrosis.

N.P.V. and Y.L. contributed equally to this research.

Accumulating evidence suggests that the renin-angiotensin system (RAS) plays an important role in the pathogenesis of DN [2, 3]. Studies showed that the renin inhibitors (aliskiren), angiotensin-converting enzyme (ACE) inhibitors, or angiotensin II type 1 (AT1) receptor antagonists ameliorate renal damage in diabetic animal models [4, 5] or slow disease progression in humans [6]. These beneficial effects of RAS inhibitors in the prevention of diabetic renal disease suggest that angiotensin II, acting through the AT1 receptor, is a major mediator of progressive renal injury. Angiotensin II has many actions that might contribute to DN [3], most prominently stimulating extracellular matrix protein synthesis in the kidney through induction of transforming growth factor- β (TGF- β) expression [7, 8].

In addition to the systemic RAS, the kidney possesses a complete intrarenal RAS [3]. The intrarenal RAS is regulated independently from the systemic RAS. In general, circulating components of the RAS are normal or suppressed in diabetic patients [9, 10]. In contrast, considerable evidence suggests that the intrarenal RAS is activated under diabetic conditions [11–13]. In addition to mesangial cells and podocytes, proximal tubular cells have been shown to express all components of the RAS and have been suggested to contribute to DN [14].

Upstream stimulatory factor 2 (USF2) was initially characterized as a transcription factor implicated in the regulation of the adenovirus major late promoter [15]. USF2 belongs to the Myc family of transcription factors and binds to the E-boxes of target genes [16]. We have previously demonstrated that USF2 expression in mesangial cells is upregulated by exposure to high glucose through the PKG, PKC, p38 MAPK and ERK pathways and mediates high-glucose-induced thrombospondin 1 (TSP1) gene expression and TGF- β production [17]. We also demonstrated that high glucose upregulates USF2 gene transcription in mesangial cells through a cAMP response element-binding protein (CREB)-dependent transactivation of the USF2 gene promoter [18]. Moreover, overexpression of USF2 stimulates TSP1 expression, TGF- β activity and extracellular matrix protein expression in the glomeruli, and accelerates the development of DN [19]. All these studies suggest that USF2 is a regulator of diabetic glomerular fibrosis. However, it is not known whether glucose regulates USF2 expression in renal proximal tubular cells and contributes to the development of diabetic tubulointerstitial fibrosis.

In the present study, we determined the effect of high glucose on USF2 expression in human renal proximal tubular cells (HK-2 cells). Our results demonstrated that

high glucose upregulated USF2 expression in HK-2 cells, which was mediated by AT1 receptor-dependent enhanced CREB phosphorylation.

Materials and Methods

Materials

Antibodies used in this study included anti-USF2, anti-ACE, and anti-actin antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif., USA), anti-phospho-CREB and anti-CREB antibodies (Cell Signaling, Danvers, Mass., USA), anti-collagen type IV antibody (Research Diagnostics, Flanders, N.J., USA), anti-fibronectin antibody (Life Technologies, Gaithersburg, Md., USA), anti-renin antibody (RDI, Concord, Mass., USA), and anti-angiotensinogen antibody (R&D System, Minneapolis, Minn., USA). Secondary antibodies were purchased from Jackson Immuno Research Laboratories, Inc. (West Grove, Pa., USA). Cell culture media were purchased from Invitrogen (Carlsbad, Calif., USA). Angiotensin II was obtained from Merck. Losartan was purchased from Sigma (St. Louis, Mo., USA). Constructs for USF2 siRNA and control siRNA were provided by Dr. McCance at University of Rochester [20].

Cell Culture

HK-2 cell line derived from normal kidney was purchased from ATCC (Manassas, Va., USA). These cells were cultured in growth medium containing DMEM/F-12, 5% FBS, penicillin (100 units/ml), streptomycin (100 μ g/ml), insulin (10 μ g/ml), transferrin (5.5 μ g/ml), selenite (5 ng/ml), dexamethasone (4 μ g/ml), glucose (5 mM), sodium pyruvate (0.5 mM), glutamine (2.5 mM), and EGF (1 ng/ml). HK-2 cells between passages 8 and 10 were used in this study. HK-2 cells were made quiescent by incubation in serum-free DMEM/F12 medium with 5 mM glucose for 48 h. All the following experiments were performed in quiescent HK-2 cells.

Real-Time PCR Assay

Quiescent HK-2 cells were treated with normal-glucose (5 mM) or high-glucose medium (30 mM) for 24 h. Mannitol (25 mM mannitol $\pm$ 5 mM glucose) was used as osmolarity control. After treatment, cells were harvested and total RNA was extracted using RNeasy mini kit, according to the manufacturer's instructions (Qiagen, Valencia, Calif., USA). Total RNA was converted to cDNA with murine leukemia virus reverse transcriptase (Promega) and real-time PCR was performed to determine the changes in USF2 and the components of the RAS as described previously [18, 21]. Primers were synthesized by Integrated DNA Technologies. The sequences are shown in table 1.

Immunoblotting

Quiescent HK-2 cells were treated with serum-free DMEM/F12 medium containing 5 mM or 30 mM glucose for 24 h in the presence or absence of losartan (1 μ M). After treatment, conditioned media were collected, loaded to 8% SDS-PAGE gel, and transferred to nitrocellulose membranes to detect fibronectin protein levels using anti-fibronectin antibody as described previously and normalized to cell numbers [22]. In addition, cells were harvested after treatments. An equal amount of protein in cell

Table 1. Primers used for real-time PCR analysis

Gene name	Oligonucleotide sequences for forward and reverse primers	Location of product, bp	GeneBank accession No.
Angiotensinogen	F: 5'-TGCTGCATGGAGTGAGCAGTAGAA-3' R: 5'-CACAAACAAGCTGGTCGGTTGGAA-3'	2145–2168 2300–2323	NM_000029
Renin	F: 5'-TCGTCTTTGACACTGGTTTCGTCCA-3' R: 5'-AGCCACTGACTGTCCCTGTTGAAT-3'	346–369 513–536	NM_000537
ACE	F: 5'-TCCTGTTACCCGAAACGAAACCCA-3' R: 5'-TCATGGAAGTGGAACTGCAGGACA-3'	1534–1557 1647–1670	NM_000789
USF2	F: 5'-TTCCGCACAGAGACAAATGGAGGA-3' R: 5'-CTGCCACCATTGCTGAAGGGATT-3'	311–334 573–596	NM_003367

extracts from different treatments was subjected to SDS-PAGE and transferred to nitrocellulose membranes to detect USF2, collagen type IV, phospho-CREB, total CREB, renin, ACE, and angiotensinogen levels with relevant antibodies. The enhanced chemiluminescence detection system (Pierce) was used for visualization of immunoreactive bands. β -Actin was used as a loading control. Immunoblots were analyzed by scanning densitometry and quantified by Quantity One gel analysis (Bio-Rad). Phospho-CREB levels were normalized to total CREB levels.

For USF2 gene knockdown studies, HK-2 cells were transiently transfected with 0.8–1 μ g of expression vectors for human USF2 siRNA or control siRNA [20] using Lipofectamine 2000 transfection reagent (Invitrogen). After 48 h, cells were treated with 5 mM or 30 mM glucose for 24 h. Fibronectin levels in the conditioned media or type IV collagen levels in the cell lysates were determined as described above by immunoblotting. The efficient USF2 knockdown in HK-2 cells was confirmed by immunoblotting.

USF2 Promoter Luciferase Assay

HK-2 cells were seeded into 6-well plates and transiently transfected with 1 μ g of USF2 promoter luciferase reporter plasmid [pGL-3-USF2 (-2400)] using Effectene transfection reagent (Qiagen) as described previously [18]. pRL-SV40 (0.02 μ g) was used as an internal control for transfection. Transfected cells were treated with normal or high glucose in the presence or absence of losartan (1 μ M) for 24 h, and the luciferase activity was assayed using the dual-luciferase assay kit (Promega).

Renin Activity and ACE Activity Assay

Quiescent HK-2 cells were treated with serum-free DMEM medium containing 5 mM or 30 mM glucose for 24 h. After treatment, conditioned media were collected. Renin activity in the conditioned media was measured using the Fluorimetric Sensolyte Renin Assay kit (Anaspec, San Jose, Calif., USA), according to the manufacturer's instructions. In addition, ACE activity in the conditioned media was measured using an ACE colorimetric assay kit (Alpco, Salem, N.H., USA).

Angiotensin II Measurement

Quiescent HK-2 cells were treated with serum-free DMEM medium containing 5 mM or 30 mM glucose for 24 h. After treat-

ment, conditioned media were collected. Angiotensin II levels in the conditioned media were determined with a commercial ELISA kit (Peninsula Laboratories) as described previously [23]. The specificity of angiotensin II ELISA for angiotensin II was tested previously [12].

Statistical Analysis

Data are expressed as means $\pm$ SE. Statistical evaluation of the data was performed using ANOVA or Student's t test as appropriate, considering $p < 0.05$ as significant.

Results

High Glucose Stimulated USF2 Expression and Increased Extracellular Matrix Accumulation (Fibronectin and Type IV Collagen) in HK-2 Cells, Which Was Abolished by siRNA-Mediated USF2 Knockdown

The effect of glucose on the expression of USF2 and extracellular matrix proteins in HK-2 cells was determined. High-glucose (30 mM glucose) treatment significantly increased USF2 protein (fig. 1a) and mRNA levels (fig. 1b). Mannitol had no effect on USF2 expression, suggesting that glucose stimulation of USF2 expression is independent of osmotic changes. In addition, fibronectin and collagen type IV levels were also upregulated by high-glucose treatment (fig. 1c, d); this effect was abolished by siRNA-mediated USF2 gene knockdown (fig. 2a, b). The efficient knockdown of USF2 by siRNA was confirmed by immunoblotting (fig. 2c). Together, these data indicate that high glucose upregulates USF2 expression in HK-2 cells. Moreover, USF2 mediates glucose-induced extracellular matrix accumulation in HK-2 cells, which may play a role in diabetic tubulointerstitial fibrosis.

Fig. 1. High glucose stimulated USF2, fibronectin and type IV collagen expression in HK-2 cells. Quiescent HK-2 cells were treated with serum-free DMEM/F12 medium containing 5 mM (NG) or 30 mM glucose (HG) for 24 h. After treatment, conditioned media were collected. The cells were harvested. USF2 protein (**a**) and mRNA levels (**b**) were determined by real-time PCR and immunoblotting, respectively. Protein levels of fibronectin (**c**) in the

conditioned medium or collagen type IV (**d**) in the cell lysates were determined by immunoblotting. The blots shown are representative of 3 separate experiments. Relative USF2, fibronectin and collagen type IV levels were determined by scanning densitometry of immunoblots. Results are means $\pm$ SE. * $p < 0.05$ vs. 5 mM glucose.

Fig. 2. siRNA-mediated USF2 gene knockdown inhibited glucose stimulation of fibronectin and type IV collagen expression in HK-2 cells. HK-2 cells were transiently transfected with control vector or USF2 siRNA constructs. After 48 h of transfection, cells were treated with normal (NG)- or high-glucose (HG) medium for 24 h. After treatment, conditioned media were collected and the cells were harvested. Protein levels of fibronectin in the con-

ditioned media (**a**) or collagen type IV (**b**) in the cell lysates were determined by immunoblotting. The blots shown are representative of three separate experiments. Relative fibronectin and collagen type IV levels were determined by scanning densitometry of immunoblots. The efficient knockdown of USF2 was confirmed by immunoblotting (**c**). Results are means $\pm$ SE. * $p < 0.05$ vs. NG; # $p < 0.05$ vs. HG (vector control).

Fig. 3. High-glucose-activated renin-angiotensin system in HK-2 cells. Quiescent HK-2 cells were treated with 5 mM (NG) or 30 mM (HG) glucose medium for 24 h. After treatment, conditioned media were collected and cells were harvested. **a** mRNA levels of angiotensinogen (AGT), renin, and ACE were analyzed by real-time PCR. **b** Protein levels of angiotensinogen, renin, and ACE were analyzed by immunoblotting. The blots shown are representative of 3 separate experiments. Relative angiotensinogen, renin and ACE protein levels were determined by scanning densitometry of immunoblots. Activity of renin and ACE (**c**) and angiotensin II levels in the conditioned media (**d**) was measured by commercially available kits as described in the Materials and Methods section. Results are means $\pm$ SE. * $p < 0.05$ vs. normal glucose.

High Glucose Upregulated Renin and Angiotensinogen Expression, and Increased Renin Activity and Angiotensin II Formation in HK-2 Cells

Proximal tubular cells have been shown to express all components of the RAS [14]. High-glucose treatment stimulated the expression of renin and angiotensinogen at both mRNA and protein levels in HK-2 cells (fig. 3a, b). In addition, the mRNA abundance of other components of RAS, including ACE (fig. 3a, b), ACE2, AT1, and AT2 receptor, was not altered by exposure to high glucose (data not shown). Consistently, renin activity in HK-2 cells was also significantly increased (fig. 3c). However, ACE activity in the conditioned media (fig. 3c) or in the cell lysates

(data not shown) was not altered by high-glucose treatment. Angiotensin II concentrations in the conditioned medium from high-glucose-treated HK-2 cells were significantly increased compared with medium from normal-glucose-treated cells (fig. 3d). Together, these studies suggest that high glucose activates RAS in HK-2 cells.

Angiotensin II Mediated High-Glucose Stimulation of USF2 Expression in HK-2 Cells through the AT1 Receptor

To test the possibility that activation of RAS in HK-2 cells mediates high-glucose-induced USF2 expression, HK-2 cells were treated with losartan, a specific AT1 an-

Fig. 4. Angiotensin II mediated high-glucose stimulation of USF2 expression in HK-2 cells. HK-2 cells were transiently transfected with USF2 promoter luciferase construct and then treated with normal (NG) or high (HG) glucose (a–c) or angiotensin (Ang) II (d–f) in the presence or absence of losartan (1 μM) for 24 h. Then cells were harvested. Protein levels of USF2 in cell lysates (a, d, e) and USF2 mRNA levels (b) were determined by immunoblotting and real-time PCR, respectively. USF2 promoter activity (c, f) was

determined by luciferase assay and normalized to Renilla luciferase level. The experiments were repeated three times and the representative data are shown. Relative levels of USF2 were determined by scanning densitometry of immunoblots. Results are means of 3 experiments ± SE. * p < 0.05 vs. normal glucose; # p < 0.05 vs. high glucose (control); [○] p < 0.05 vs. control; [▼] p < 0.05 vs. angiotensin II treatment.

tagonist, prior to normal- or high-glucose treatment. High-glucose stimulation of USF2 protein levels (fig. 4a), mRNA levels (fig. 4b), and promoter activity (fig. 4c) was prevented by losartan treatment. In addition, angiotensin II treatment dose dependently stimulated USF2 expression in HK-2 cells in normal-glucose medium (fig. 4d). The effect of angiotensin II on USF2 expression – protein levels (fig. 4e) or promoter activity (fig. 4f) – was inhibited by losartan treatment. Together, these data suggest

that angiotensin II mediates high-glucose-induced USF2 expression in HK-2 cells, possibly through the AT1 receptor.

High Glucose Activated the Transcription Factor CREB in HK-2 Cells, Which Was Inhibited by Losartan Treatment

We have previously shown that high glucose upregulates USF2 gene transcription in mesangial cells through

Fig. 5. Losartan treatment inhibited high-glucose-increased CREB phosphorylation in HK-2 cells. HK-2 cells were treated with normal (NG) or high (HG) glucose in the presence or absence of losartan (1 μ M) for 24 h. Levels of the phospho-CREB (Ser133) in cell lysates were analyzed by immunoblotting and normalized to total CREB levels. The experiments were repeated 3 times and the representative data are shown. Relative levels of USF2 or phospho-CREB were determined by scanning densitometry of immunoblots. Results are means $\pm$ SE of 3 experiments. * $p < 0.05$ vs. normal glucose; # $p < 0.05$ vs. high glucose (control).

a CREB-dependent transactivation of the USF2 gene promoter [18]. In this study, similarly, we found that 24 h of high-glucose exposure increased the levels of CREB phosphorylation (reflecting CREB activation) in HK-2 cells. Moreover, the glucose-mediated increase in CREB phosphorylation was inhibited by losartan treatment (fig. 5), suggesting that high-glucose stimulation of USF2 expression in HK-2 cells is partially mediated by angiotensin II-AT1-dependent CREB activation.

Discussion

Our studies provide the first evidence that angiotensin II mediates glucose-induced USF2 expression in renal proximal tubular cells through AT1 receptor-dependent activation of the transcription factor CREB. Importantly, siRNA-mediated USF2 knockdown abolishes high-glucose-induced extracellular matrix accumulation in proximal tubular cells, supporting a role for USF2 in the development of diabetic tubulointerstitial fibrosis.

We have previously demonstrated that USF2 expression in mesangial cells was upregulated by high-glucose exposure through the PKG, PKC, p38 MAPK, and ERK pathways and mediated high-glucose-induced TSP1 gene expression and TGF- β production [17]. We also demonstrated that high glucose upregulated USF2 gene transcription in mesangial cells [18]. By using the luciferase promoter deletion assay, site-directed mutagenesis, ChIP assay, and transactivation assay, we identified a glucose-responsive element in the USF2 gene promoter and demonstrated that glucose-induced USF2 expression was mediated through CREB-dependent transactivation of the USF2 promoter. Moreover, overexpression of USF2 stimulated TSP1 expression, TGF- β activity and extracellular matrix protein expression in the glomeruli, and accelerated the development of DN in vivo in a type 1 diabetic mouse model [19]. All these studies suggest that USF2 is a regulator of diabetic glomerular fibrosis. In the present studies, we demonstrated that high glucose also upregulated USF2 expression (mRNA, protein levels and promoter activity) in renal proximal tubular cells. Furthermore, USF2 mediated high-glucose stimulation of extracellular matrix accumulation (fibronectin and collagen IV) in proximal tubular cells, suggesting that USF2 is also involved in the development of tubulointerstitial fibrosis under diabetic conditions. Taken together, our previous and current studies provide strong evidence for the role of USF2 in the development of DN. Importantly, our studies may lead to the development of novel therapies to ameliorate this major complication of diabetes.

Accumulating evidence suggests that the intrarenal RAS is activated under diabetic conditions and plays an important role in DN [11–13]. It has been shown that kidney proximal tubular cells express all components of the RAS [14]. By using HK-2 cells, we demonstrated that high-glucose treatment stimulated the expression of both renin and angiotensinogen. Consistently, renin activity was increased, leading to increased formation of angiotensin II in these cells. Our result is consistent with the studies of Hsieh et al. [24] showing that glucose similarly upregulated renin and angiotensinogen expression in rat kidney proximal tubular cells. In contrast, Feliers and Kasinath [25] failed to observe the increased renin expression in high-glucose-treated murine proximal tubular cells. This difference might be due to the different species of cells or different cultural conditions.

The mechanisms by which glucose stimulates angiotensinogen expression in proximal tubular cells have been investigated. Studies by Hsieh et al. [24] demonstrated that activation of the hexosamine biosynthesis path-

way is involved in glucose stimulation of angiotensinogen expression in rat kidney proximal tubular cells. Angiotensin II has also been shown to stimulate angiotensinogen expression in both rat and human renal proximal tubular cells [26, 27]. However, the mechanisms by which glucose upregulates renin expression in tubular cells are not known. We have previously demonstrated that USF2 binds to the renin gene promoter to stimulate renin expression in mesangial cells [21]. This could result in a feedback loop that may amplify the effects of high glucose on renin expression and then the activation of endogenous RAS in human renal proximal tubular cells.

As discussed above, endogenous RAS in human proximal tubular cells was activated by high-glucose treatment. By using the AT1 receptor antagonist losartan, we demonstrated that increased angiotensin II levels mediate glucose-induced USF2 expression through the AT1 receptor. Moreover, we found that high glucose activated CREB in proximal tubular cells, which was inhibited by losartan treatment, suggesting that glucose stimulation of USF2 expression in proximal tubular cells is partially mediated by angiotensin II-AT1-dependent CREB acti-

vation. It is known that CREB is a cAMP response element-binding protein and mediates glucose-induced USF2 expression in mesangial cells [18]. At this time, the signaling pathways leading to angiotensin II-AT1-dependent CREB activation in proximal tubular cells are not known and need to be further investigated.

In conclusion, our studies demonstrated that high glucose activates the endogenous RAS in human renal proximal tubular cells, which mediates glucose stimulation of USF2 expression through angiotensin II-AT1 receptor-dependent activation of the transcription factor CREB. Moreover, USF2 mediates high-glucose stimulation of extracellular matrix protein accumulation in proximal tubular cells, suggesting an important role for USF2 in the development of diabetic tubulointerstitial fibrosis.

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