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Transcription Factors Krüppel-Like Factor 6 and Peroxisome Proliferator-Activated Receptor-γ Mediate High Glucose-Induced Thioredoxin-Interacting Protein

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We demonstrated recently that thioredoxin-interacting protein (Txnip) and the transcription factor Krüppel-like factor 6 (KLF6) were up-regulated in both *in vivo* and *in vitro* models of diabetic nephropathy, thus promoting renal injury. Conversely, peroxisome proliferator-activated receptor-γ (PPAR-γ) agonists have been shown to be renoprotective. Hence, this study was undertaken to determine whether Txnip expression is regulated by the transcription factors KLF6 and PPAR-γ. By using siRNAs and overexpressing constructs, the role of KLF6 and PPAR-γ in Txnip transcriptional regulation was determined in human kidney proximal tubule cells and in streptozocin-induced diabetes mellitus in Sprague-Dawley rats, *in vitro* and *in vivo* models of diabetic nephropathy, respectively. KLF6 overexpression increased Txnip expression and promoter activity, which was inhibited by concurrent exposure to PPAR-γ agonists. In contrast, reduced expression of KLF6 by siRNA or exposure to PPAR-γ agonists attenuated high glucose-induced Txnip expression and promoter activity. KLF6-Txnip promoter binding was decreased in KLF6-silenced cells, whereas PPAR-γ agonists increased PPAR-γ-Txnip promoter binding. Indeed, silencing of KLF6 increased PPAR-γ expression, suggesting endogenous regulation of PPAR-γ expression by KLF6. Moreover, renal KLF6 and Txnip expression increased in rats with diabetes mellitus and was inhibited by PPAR-γ agonist treatment; however, KLF6 expression did not change in HK-2 cells exposed to PPAR-γ agonists. Hence, Txnip expression and promoter activity are mediated via divergent effects of KLF6 and PPAR-γ transcriptional regulation. ***(Am J Pathol 2009, 175:1858–1867; DOI: 10.2353/ajpath.2009.090263)***

Thioredoxin-interacting protein (Txnip), also known as vitamin D-up-regulated protein-1 (VDUP1) and thioredoxin-binding protein-2 (TBP-2), was initially found to be up-regulated in HL-60 cells treated with 1α,25-dihydroxyvitamin D_3.[1] Txnip is known to play various roles in proliferation, apoptosis, redox signaling, vascular inflammation, and lipid and glucose metabolism[2–7] and hence may be considered a key mediator of the cellular and metabolic abnormalities that are central to the development of diabetes complications. Recently, elegant work by Chen et al[8] showed that Txnip deficiency protects β-cells against glucose toxicity and apoptosis. Furthermore, lack of Txnip induces pancreatic β-cell mass, thus protecting against both streptozotocin (STZ)- and obesity-induced diabetes.[9] Txnip is not only a thioredoxin inhibitor but also a critical regulator of glucose metabolism in liver and heart, and it has been demonstrated to play a key role in global glucose homeostasis.[6,10] Hyperglycemia is known to be a key causative factor in diabetes-related complications and Txnip has been shown to be markedly up-regulated in microarray studies in both INS-1 β-cells and proximal tubule kidney cells exposed to high glucose conditions.[11,12] Txnip is increased in mesangial cells exposed to high glucose, and increased levels of Txnip result in collagen accumulation, suggesting its role in the

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development of matrix accumulation, characteristic of diabetic nephropathy.[13] Our previous studies have suggested that high glucose-induced up-regulation of Txnip expression in human proximal tubule cells is independent of transforming growth factor-β1, which is also known to induce extracellular matrix production. *In vivo* models of diabetic nephropathy have similarly confirmed the up-regulation of Txnip.[12] Using Genomatix software, we have found that there are seven binding sites of Krüppel-like factor 6 (KLF6) and three binding sites of peroxisome proliferator-activated receptor-γ (PPAR-γ) located in the Txnip promoter region.[14] Hence we hypothesized that transcription factors KLF6 and PPAR-γ may also regulate Txnip expression and promoter activity in hyperglycemic conditions.

KLF6 is a DNA-binding protein containing a triple zinc-fingered motif, which plays a key role in the regulation of cell proliferation, differentiation, and development.[15,16] KLF6 is known to be expressed in embryonic kidney[17] but, interestingly, was found to be up-regulated in injured hepatic stellate cells[18] and in the kidneys of mice undergoing ischemic reperfusion injury.[19] Because embryonic transcription factors and proteins are re-expressed in renal injury,[20] we initially explored whether KLF6 expression is recapitulated in diabetic nephropathy. Indeed, our recent study demonstrated that KLF6 was induced by high glucose in HK-2 cells and up-regulated in the kidney of the diabetic Ren-2 rat, which exhibits significant nephropathy.[21] Furthermore, KLF6 was demonstrated to play an important role in transforming growth factor-β-induced epithelial to mesenchymal transition in HK-2 cells.[21]

PPARs are ligand-activated nuclear receptors and transcription factors. PPARs have attracted enormous attention in the past decade as a result of their therapeutic roles in metabolic diseases such as diabetes and obesity.[22,23] There are three isoforms of PPARs: PPAR-α, PPAR-β/δ, and PPAR-γ. PPAR-γ has been a large focus of PPAR study because it is a key factor in adipogenesis, insulin sensitivity, cell cycle regulation, and cell differentiation.[24–26] The thiazolidinediones pioglitazone and rosiglitazone are potent PPAR-γ agonists that have been used in the treatment of patients with type II diabetes.[27,28] Our previous work confirms that PPAR-γ is present in proximal tubule kidney cells and inhibits high glucose-induced inflammation.[29] Because both PPAR-γ and Txnip play key roles in glucose homeostasis, we further hypothesized a link between PPAR-γ and Txnip under high glucose conditions. Hence, in the current study we aimed to determine the roles of KLF6 and PPAR-γ in regulating high glucose-induced Txnip expression and promoter activity.

Materials and Methods

Cell Culture

Human kidney-2 (HK-2) cells, an immortalized human kidney proximal tubule cell line from American Type Cell Collection (Rockville, MD), were used in this study. Cells were grown in keratinocyte serum-free media (Invitrogen, Carlsbad, CA) as described previously[30–32] and seeded at 80 to 90% confluence before different cell culture treatments. Cells were exposed to the following treatment conditions: 5 mmol/L D-glucose (normal glucose, HK-2 cells culture media), 30 mmol/L D-glucose (high glucose), 2 or 10 μmol/L of vehicle (dimethyl sulfoxide [DMSO], Sigma-Aldrich, St. Louis, MO), pioglitazone (Alexis Chemicals, San Diego, CA), and rosiglitazone (Cayman Chemical, Ann Arbor, MI). The concentrations of pioglitazone and rosiglitazone and the period of study are indicated in the figure legends.

Small Interference RNA

Small interference (si) RNAs were designed and chemically synthesized (Qiagen, Valencia, CA). The targeting mRNA sequences for KLF6 and PPAR-γ are 5′-AAGCCAGGTGACAAGGGAAATGGCGAT-3′ and 5′-GCCTCATGAAGAGCCTTCCAACTCCCTCA-3′, respectively. HK-2 cells were seeded at 90% confluence, and siRNAs were introduced using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. In parallel, cells were transfected with nonspecific siRNA, which served as the control. Silencing efficiency of these two siRNAs reached 60 to 90% as described previously.[21]

KLF6 Overexpression Plasmid Construct

To enforce KLF6 expression in the cells, the full-length cDNA (852 bp) of human KLF6 (NM 001300) containing NheI and XhoI restriction digestive sites at both ends was amplified using a PfuUltra Hotstart DNA Polymerase Kit (Stratagene, La Jolla, CA). The amplified KLF6 DNA fragment was subsequently cloned into pcDNA3.1 (Invitrogen). KLF6 plasmids were purified using a PureYield Plasmid Midiprep System (Promega, Madison, WI). HK-2 cells were seeded at 90% confluence, and 4 μg of KLF6 construct or construct containing no targeting sequence (empty vector) were introduced into HK-2 cells using Lipofectamine 2000 on six-well culture dishes as described previously.[21]

Real-time RT-PCR

Real-time PCR was used to assess transcript levels with negative controls included in each run. Specific primers for the use of SYBR Green are shown in Table 1. Primer specificity in real-time PCR reactions was confirmed using RT-PCR. In brief, total RNA (1 μg) was treated with DNase I (Invitrogen), and then RNA was used in 25 μl of a real-time PCR reaction including Brilliant SYBR Green One-Step QRT-PCR Master Mix according to the manufacturer's instructions (Stratagene). Real-time quantitations were performed on the Bio-Rad iCycler iQ system (Bio-Rad Laboratories, Hercules, CA). The fluorescence threshold value was calculated using the iCycle iQ system software. The calculation of relative change in mRNA was performed using the ΔΔ method as described previously,[31,33,34] with normalization for the housekeeping gene 18S. RT-PCR was also performed

Table 1. RT-PCR Primers

Gene name	Accession no.	Sense	Antisense	Size (bp)
Human *TXNIP*	NM_006472	5′-CGCCACACTTACCTTGCCAATG-3′	5′-GCTCTTGCCACGCCATGATG-3′	111
Human *KLF6*	NM_001300	5′-TCCACGCCTCCATCTTCT-3′	5′-CATCGCCATTTCCCTTGT-3′	136
Human *PPAR-γ*	NM_138711	5′-CAAAGCAAAGGCGAG-3′	5′-ACGGAGCGAAACTGGC-3′	170
Human *18S*	NR_003286	5′-CGGCTACCACATCCAAGGAA-3′	5′-GCTGGAATTACCGCGGCT-3′	186
Rat *TXNIP*	NM_001008767	5′-AGGATTCTGTGAAGGTGATG-3′	5′-TCTGACTGAGGACAGCTTCT-3′	154
Rat *KLF6*	NM_031642	5′-GTAGGCTAAAAGAGGCTTCC-3′	5′-TAGAAACCAGTGGTGAGTCC-3′	208
Rat *PPAR-γ*	NM_013124	5′-TACCATGGTTGACACAGAGA-3′	5′-AACGGGATGTCTTCATAGTG-3′	162
Rat *18S*	EU637075	5′-TCGAGGCCCTGTAATTGGAAA-3′	5′-CCCTCCAATGGATCCTCGTT-3v	85

to measure Txnip and β-actin mRNA expression using a PCR kit from Qiagen. Amplification products were electrophoresed through 1.5% (w/v) agarose gels and visualized by ethidium bromide staining.

Western Blotting

The method for detecting Txnip, KLF6s and PPAR-γ protein levels using Western blotting has been described previously.[12] In brief, 30 μg of protein from each sample was subjected to SDS-polyacrylamide gel electrophoreses. Proteins were then transferred to Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK). Nonspecific binding sites were blocked for 1 hour (5% nonfat milk and 0.1% Tween 20 in PBS) after which the membranes were exposed to Txnip (1:400 dilution, ZYMED Laboratories, Carlsbad, CA), KLF6 (1:200 dilution, Santa Cruz Biotechnology, Santa Cruz, CA), or PPAR-γ (1:300 dilution, Santa Cruz Biotechnology) antibodies in 5% nonfat milk and 0.1% Tween 20 in PBS overnight at 4°C, followed by washing three times after which they were incubated with peroxidase-labeled secondary antibodies (Amersham Pharmacia Biotech) for 1 hour and again washed three times. The blots were then detected using ECL (Amersham Pharmacia Biotech). The bands corresponding to Txnip (48 kDa), KLF6 (46 kDa), and PPAR-γ (55 kDa) were quantitated using Gel Documentation (Bio-Rad Laboratories). The blots were then reprobed with tubulin antibody (1:1000 dilution, 55 kDa, Abcam, Cambridge, UK) or actin (1:500 dilution, 42 kDa, NeoMarkers, Fremont, CA) as a loading control.

Txnip Promoter Activity Assay

The promoter activity of Txnip was determined by the Dual-Luciferase Reporter Assay System (Promega) as described previously.[12] In brief, the promoter sequence of Txnip was designed and amplified using a GC-2 PCR kit (BD Biosciences, San Diego, CA). The DNA fragment of Txnip promoter was cloned into pGL3 firefly luciferase vector (Promega). The plasmid containing the Txnip promoter sequence was introduced into HK-2 cells using Lipofectamine 2000 (Invitrogen). pRL-SV40 *Renilla* vector (Promega) was cotransfected into cells, and its luciferase activity was used for normalization of transfection efficiency. Luciferase activity was detected by POLARstar (BMG, Labtech, Offenburg, Germany).

Chromatin Immunoprecipitation Assay

A chromatin immunoprecipitation (ChIP) assay was performed using an EZ ChIP kit (Upstate, Kankakee, IL) according to the manufacturer's instructions. In brief, cells were cross-linked in 1% formaldehyde after different cell culture treatments. Cells were then lysed and sonicated. Sonication was optimized to achieve 200- to 1000-bp DNA fragments. Equal amounts of protein were immunoprecipitated with antibodies detecting KLF6 and PPAR-γ (Santa Cruz Biotechnology) at concentrations of 1 μg/ml, respectively. Immunoprecipitated protein-DNA cross-linked samples were then reversed, and DNA samples were purified using spin columns. PCR was performed using specific primers on the above-purified DNA samples. PCR products were run on 2% agarose gel, and the bands were quantitated using Gel Documentation (Bio-Rad Laboratories). ChIP primers detecting the KLF6 or PPAR-γ binding region in the Txnip promoter are described in Table 2.

In Vivo *Studies in Diabetic Rats*

Six-week-old male Sprague-Dawley rats were randomized to receive either 55 mg/kg of STZ (Sigma-Aldrich) diluted in 0.1 mol/L citrate buffer, pH 4.5, or citrate buffer (nondiabetic) by tail vein injection after an overnight fast as described previously.[35] Blood glucose was determined using an AMES glucometer (Bayer Diagnostics, Melbourne, VIC, Australia), and only STZ-treated animals with blood glucose >20 mmol/L were studied. Diabetic rats were divided into three groups: one gavaged with 40 mg/kg/day pioglitazone (Eli Lilly, Indianapolis, IN), one gavaged with 5 mg/kg/day rosiglitazone (GlaxoSmithKline, Brentford, Middlesex, UK), and one gavaged with vehicle (water) for 3 weeks. At the end of the 3-week study (at age

Table 2. Chromatin Immunoprecipitation Assays Primers

Binding region in Txnip promoter	Sense	Antisense
KLF6	5′-GGTCAGTGGGATCCTCCTTC-3′	5′-GAAAATGGTTGTTGCGCTCT-3′
PPAR-γ	5′-AAACACGCCCCTCCTATTTC-3′	5′-TCAGGCCTCATTGTGTGTGT-3′

of 9 weeks), kidney cortex tissues were dissected and snap-frozen for RNA extraction using TRIzol (Invitrogen). All animals were housed in a stable environment maintained at 22 ± 1°C with a 12-hour light/dark cycle. Experimental procedures adhered to the guidelines of the National Health and Medical Research Council of Australia's Code for the Care and Use of Animals for Scientific Purposes and were approved by the Animal Research Ethics Committee of St. Vincent's Hospital.

Statistical Analysis

All results are expressed as a fold change compared with the control value. Each experiment was performed independently a minimum of three times in cell culture or otherwise stated in figure legends and six animals in each group were used in the *in vivo* study. Results are expressed as means ± SEM. Statistical comparisons between groups were made by analysis of variance, with pairwise multiple comparisons made by Fisher's protected least-significant difference test. Analyses were performed using the software package, Statview (version 4.5, Abacus Concepts Inc., Berkley, CA). $P < 0.05$ was considered significant.

Results

High Glucose-Induced Txnip Expression and Promoter Activity Are Mediated via KLF6

We demonstrated previously that high glucose induced KLF6 expression and dramatically up-regulated Txnip in HK-2 cells.[12,21] In this study, we first determined whether KLF6 mediates high glucose-induced Txnip mRNA and protein expression. KLF6 siRNA attenuated high glucose-induced Txnip mRNA (Figure 1A) and protein (Figure 1B) levels compared with those in cells transfected with nonspecific siRNA exposed to high glucose for 72 hours. Txnip promoter activity induced by high glucose peaked at 24 hours as reported previously[12] and KLF6 siRNA attenuated high glucose-induced Txnip promoter activity compared with that in cells transfected with nonspecific siRNA (Figure 1C). These data suggest that KLF6 mediates high glucose-induced Txnip via promoting Txnip promoter activity with subsequent increased levels of Txnip transcription and translation.

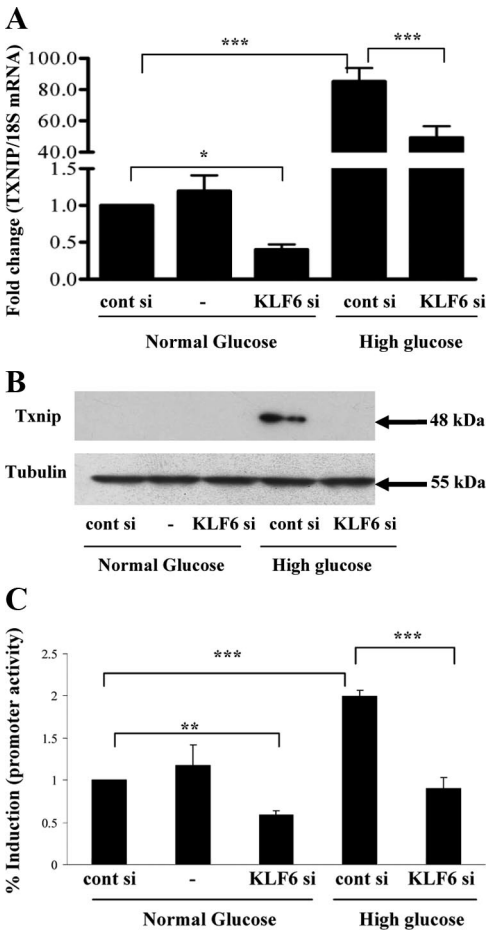

Figure 1. High glucose-induced Txnip expression and promoter activity is mediated via KLF6. HK-2 cells were transfected with 120 nmol/L nonspecific siRNA (cont si) or KLF6 siRNA (KLF6 si) or transfection reagent alone (−) using Lipofectamine 2000 on six-well culture dishes. Cells were then exposed to 5 mmol/L (normal glucose) and 30 mmol/L (high glucose) D-glucose for either 72 hours (**A:** real-time PCR; **B:** Western blotting) or 24 hours (**C:** promoter activity) after transfection. Results are means ± SEM and are shown as fold change compared with control. Three independent cell culture preparations were performed. $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0005$.

Role of PPAR-γ in High Glucose-Induced Txnip Expression

Txnip is known to be a critical glucose metabolism regulator in both liver and islet cells.[6,8] PPAR-γ is well known to regulate glucose metabolism in muscles, liver, and kidney.[36,37] Hence we determined the role that PPAR-γ plays in high glucose-induced Txnip expression. High glucose decreased PPAR-γ mRNA expression after cells were exposed to high glucose for 72 hours (Figure 2A). Both pioglitazone and rosiglitazone attenuated high glucose-induced Txnip mRNA (Figure 2B) and protein (Figure 2C) expression at 72 hours and promoter activity at 24 hours (Figure 2D). Both pioglitazone and rosiglitazone induced PPAR-γ mRNA expression at the basal level (Figure 2E). Furthermore, Txnip mRNA expression significantly increased in PPAR-γ silenced cells (Figure 2F), suggesting a direct role for PPAR-γ in regulating Txnip expression.

Role of PPAR-γ on Txnip Transcriptional Level in KLF6 Overexpressing Cells

We further determined whether Txnip mRNA expression increased in cells in which KLF6 was overexpressed and whether PPAR-γ agonists could attenuate this effect. As expected, the levels of Txnip mRNA (Figure 3A) and promoter activity (Figure 3B) increased in cells overexpressing KLF6, and this increase was inhibited by either pioglitazone or rosiglitazone. These data suggest that PPAR-γ attenuated the high glucose-induced Txnip by limiting the KLF6 induction of Txnip in HK-2 cells.

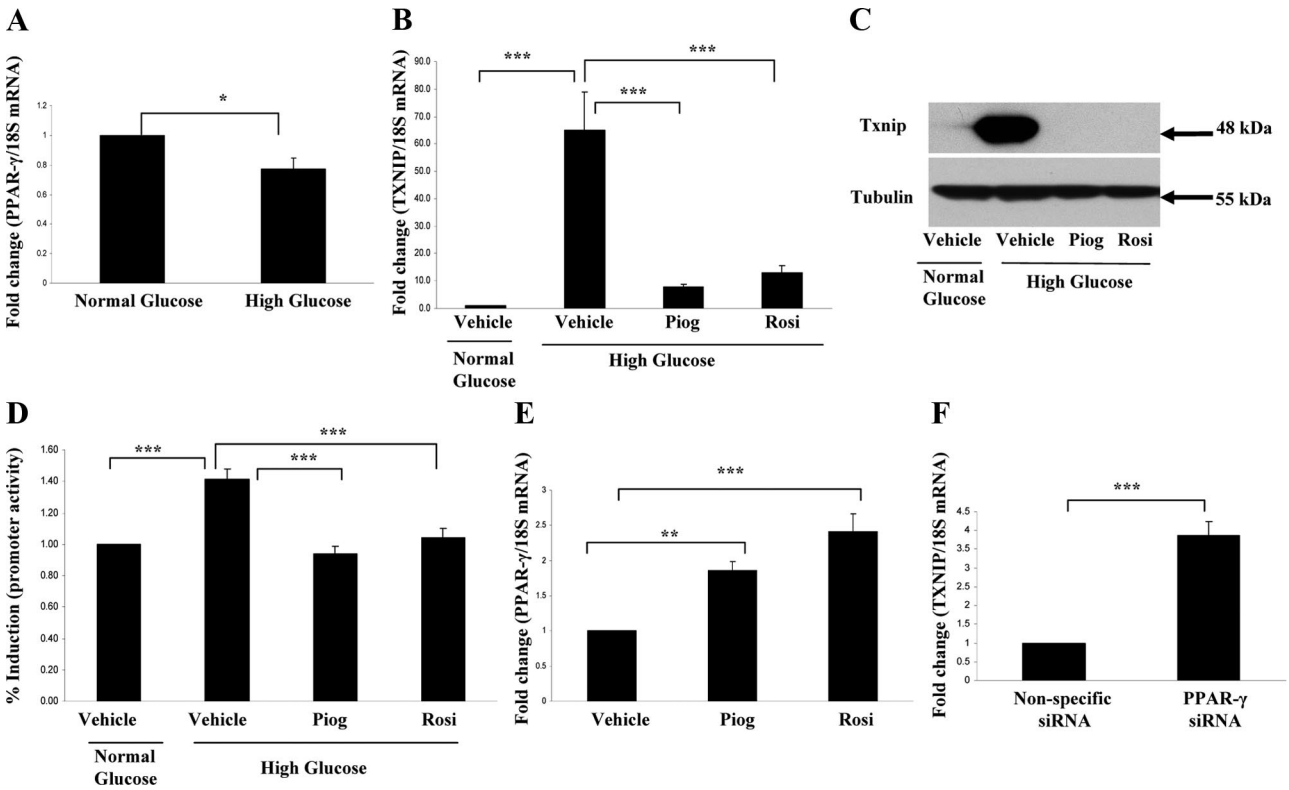

Figure 2. The role of PPAR-γ in high glucose-induced Txnip expression. **A:** HK-2 cells were exposed to 5 mmol/L (normal glucose) or 30 mmol/L (high glucose) D-glucose for 72 hours and PPAR-γ and 18S mRNA expression was measured by real-time RT-PCR. **B–D:** HK-2 cells were exposed to 5 mmol/L (normal glucose) with vehicle (DMSO), 30 mmol/L (high glucose) D-glucose with vehicle (DMSO), high glucose with 10 μmol/L pioglitazone (Piog), high glucose with 10 μmol/L rosiglitazone for 72 hours (**B:** real-time RT-PCR; **C:** Western blot) or 24 hours (**D:** promoter activity). **E:** HK-2 cells were exposed to vehicle (DMSO), 10 μmol/L pioglitazone, and 10 μmol/L rosiglitazone, respectively, for 72 hours, and PPAR-γ and 18S mRNA expression was measured by real-time RT-PCR**. F:** HK-2 cells were transfected with 120 nmol/L nonspecific (control) or PPAR-γ siRNAs using Lipofectamine 2000 on six-well culture dishes. RNA was collected after 24 hours of transfection and Txnip and 18S mRNA expression was measured by real-time RT-PCR. Results are means ± SEM and are shown as fold change compared with control. Three independent cell culture preparations were performed. $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0005$.

Reciprocal Effects of Transcription Factors PPAR-γ and KLF6

Because both transcription factors PPAR-γ and KLF6 modify high glucose-induced Txnip, we further determined whether there is a reciprocal effect between PPAR-γ and KLF6. PPAR-γ mRNA was increased in KLF6-silenced cells (Figure 4A) and decreased in KLF6-overexpressing cells (Figure 4B). However, KLF6 mRNA expression did not change in cells treated with pioglitazone or rosiglitazone under normal glucose or high glucose compared with cells treated with vehicle (Figure 4C), suggesting that KLF6 is upstream of PPAR-γ and it regulates PPAR-γ expression. We have found there is at least one KLF6 binding site on the promoter of PPAR-γ. However there is no PPAR-γ binding site on KLF6 promoter. This finding is consistent with our data and further confirms that KLF6 is upstream of PPAR-γ and regulates PPAR-γ expression.

KLF6 Regulates Txnip Expression by Binding to Txnip Promoter

Ratziu et al[14] reported that KLF6 (Zf9) binds specifically to a DNA oligonucleotide containing a GC box motif and that full-length KLF6 transactivates a reporter construct driven by the simian virus 40 promoter enhancer, which contains several GC boxes.[14] Use of Genomatix software

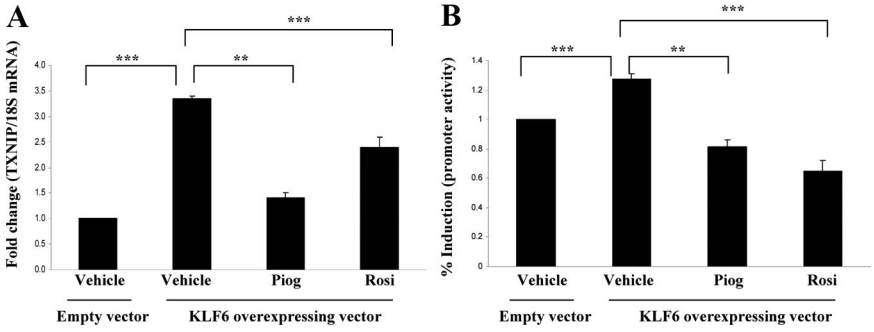

Figure 3. The role of PPAR-γ on Txnip transcriptional level in KLF6-overexpressing cells. HK-2 cells were transfected with 4 μg of empty vector or a KLF6 overexpressing plasmid construct using Lipofectamine 2000 on six-well culture dishes. Cells were then exposed to vehicle (DMSO), 10 μmol/L pioglitazone (Piog), or 10 μmol/L rosiglitazone (Rosi) for either 72 hours for the measurement of Txnip mRNA expression (**A**) or 24 hours for the measurement of Txnip promoter activity (**B**). Results are means ± SEM and are shown as fold change compared with control. Three independent cell culture preparations were performed. $^{**}P < 0.005$; $^{***}P < 0.0005$.

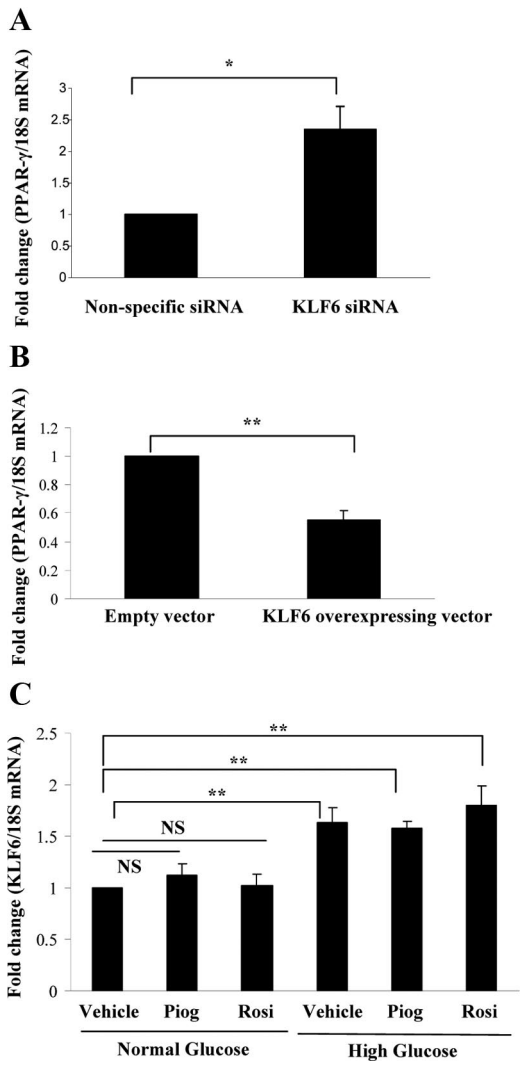

Figure 4. Reciprocal effects of PPAR-γ and KLF6. **A** and **B:** HK-2 cells were transfected with 120 nmol/L nonspecific (control) or KLF6 siRNAs (**A**) or 4 μg of empty vector or KLF6-overexpressing plasmid construct (**B**) using Lipofectamine 2000 on six-well culture dishes. RNA was collected after 24 hours transfection and PPAR-γ and 18S mRNA expression was measured by real-time RT-PCR. **C:** Cells were exposed to vehicle (DMSO), 10 μmol/L pioglitazone (Piog), or 10 μmol/L rosiglitazone (Rosi) with the treatment of normal or high glucose for 72 hours. KLF6 and 18S mRNA expression was measured by real-time RT-PCR Results are means ± SEM and shown as fold change compared with control. Three independent cell culture preparations were performed. $^{*}P < 0.05$; $^{**}P < 0.005$; NS, statistically nonsignificant.

to search transcription factor binding sites showed seven consensus binding sites (GC box) of zinc finger binding proteins including KLF6 within the promoter region of human TXNIP. A ChIP assay was performed with specific primers detecting the Txnip promoter regions to which KLF6 binds. The level of KLF6-Txnip promoter binding decreased in KLF6-silenced cells (Figure 5A) and increased in KLF6-over expressing cells (Figure 5B), confirming that KLF6 binds to Txnip promoter and positively regulates Txnip expression. Furthermore, the level of KLF6-Txnip promoter binding increased in cells treated with high glucose, and this induction was not affected by treatment with pioglitazone or rosiglitazone (Figure 5C–D), further confirming that KLF6 is upstream of PPAR-γ and hence that the attenuation of Txnip mRNA and promoter activity in the presence of thiazolidinediones is not mediated through a KLF6-dependent mechanism.

PPAR-γ Binds to Txnip Promoter and Thiazolidinediones Promote This Binding Reaction

In their review, Schoonjans et al[38] noted that PPAR protein binds to PPAR response elements. A search of the results with Genomatix software showed three PPAR consensus binding sites within the promoter region of human TXNIP. A ChIP assay was performed with specific primers detecting the Txnip promoter regions to which PPAR-γ binds. Pioglitazone or rosiglitazone significantly increased PPAR-γ-Txnip promoter binding compared with that of cells treated with the vehicle DMSO (Figure 6A). Furthermore, the level of PPAR-γ-Txnip promoter binding decreased in cells treated with high glucose, and this reduction was attenuated by the treatment of pioglitazone or rosiglitazone (Figure 6B). These data suggest that PPAR-γ binds to the Txnip promoter; however, in contrast with KLF6 binding, it negatively regulates Txnip expression.

Txnip, KLF6, and PPAR-γ Expression in Kidney Cortex Tissues in Sprague-Dawley Rats with STZ-Induced Diabetes of 3 Weeks Duration

We demonstrated previously that Txnip and KLF6 were up-regulated in the kidneys of diabetic Ren-2 rats at 16 weeks, when hypertension, albuminuria, a declining glomerular filtration rate, severe glomerulosclerosis, and tubulointerstitial disease are present.[12,21] Sprague-Dawley rats induced to develop diabetes using STZ do not develop any of the structural and functional manifestations of diabetic nephropathy after 3 weeks of hyperglycemia. Despite the lack of structural and functional manifestations of nephropathy, renal cortical mRNA expression of Txnip (Figure 7A) was up-regulated and attenuated by pioglitazone or rosiglitazone. KLF6 mRNA was up-regulated in diabetic kidney, but in contrast to the results of the *in vitro* studies, treatment with either PPAR-γ agonist reduced KLF6 mRNA expression (Figure 7B). Taken together with the *in vitro* studies, this result suggests that an unmeasured metabolic parameter influenced by PPAR-γ agonist treatment indirectly reduced KLF6 expression. In keeping with the model of STZ diabetes, PPAR-γ treatment did not result in improved glycemic control (data not shown). PPAR-γ mRNA was slightly decreased in the kidneys of diabetic rats but increased in diabetic rats treated with pioglitazone or rosiglitazone (Figure 7C). The nonsignificant statistical decrease of PPAR-γ mRNA expression in diabetic rats may be due to the mixed cell population in the kidney cortex. These data suggest that Txnip and KLF6 might be early markers indicating cellular dysfunction in diabetic conditions.

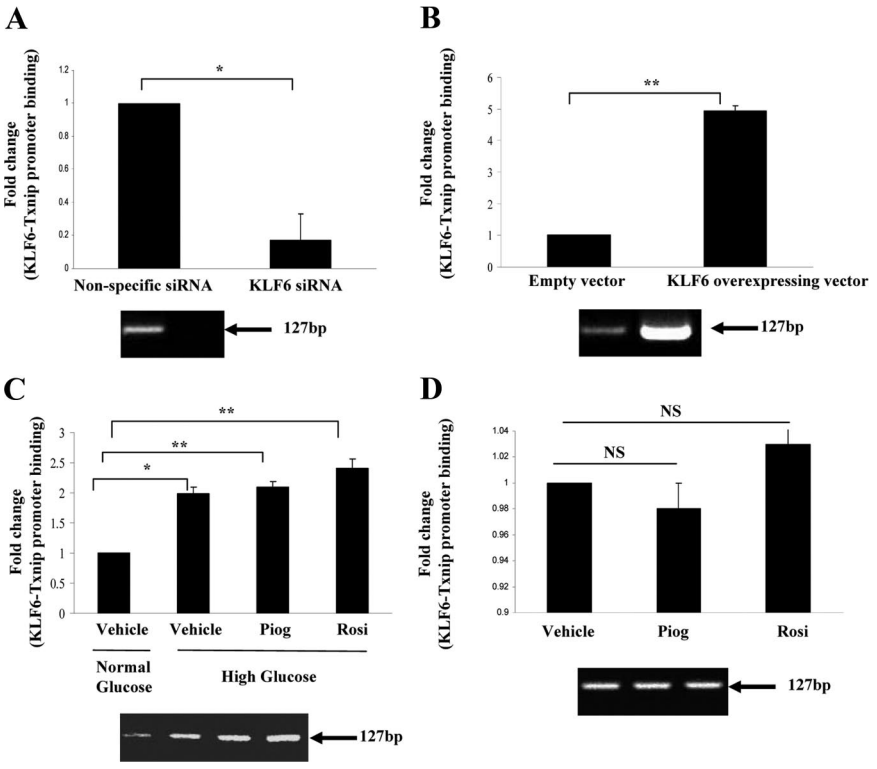

Figure 5. KLF6-Txnip promoter binding activity. HK-2 cells were transfected with 120 nmol/L nonspecific (control) or KLF6 siRNAs (**A**) or 4 μg of empty vector or KLF6-overexpressing plasmid construct (**B**) using Lipofectamine 2000. **C** and **D:** Cells were exposed to vehicle (DMSO), 10 μmol/L pioglitazone (Piog), or 10 μmol/L rosiglitazone (Rosi) with the treatment of normal or high glucose for 72 hours. A ChIP assay was performed using an EZ ChIP kit according manufacturer's instructions. RT-PCR was performed on purified DNA samples that were immunoprecipitated with KLF6 antibody. PCR products were run on 2% agarose gel, and a representative gel is shown below each graph with quantification by densitometry using Quantity One software (Bio-Rad Laboratories). Results are means ± SEM and are shown as fold change compared with control. Two independent cell culture preparations were performed. $^{*}P < 0.05$; $^{**}P < 0.005$; NS, statistically nonsignificant.

Discussion

In this study we explored the roles of KLF6 and PPAR-γ in the coordinated regulation of Txnip expression and promoter activity in the human proximal tubule under high glucose conditions and in the kidney of an *in vivo* model of diabetes mellitus. Our data demonstrate that Txnip is markedly up-regulated in response to high glucose in the kidney before structural abnormalities appear. Hence it is likely to play an important role in the dysregulated glucose metabolism and cellular abnormalities observed in the proximal tubule in diabetic nephropathy. Furthermore, we have demonstrated that KLF6 and PPAR-γ independently bind to the Txnip promoter region, with KLF6 increasing and PPAR-γ reducing Txnip expression. KLF6 mRNA expression did not change in cells treated with PPAR-γ agonists (thiazolidinediones). In contrast, PPAR-γ mRNA expression was affected by the levels of KLF6 in the cells, suggesting that PPAR-γ is downstream of KLF6 in regulating Txnip expression in high glucose conditions. Our *in vivo* study in diabetic rats with treatments of PPAR-γ agonists confirms the key roles of KLF6 and PPAR-γ in the regulation of Txnip expression very early in the development of diabetes mellitus.

The transcriptional mechanisms of Txnip regulation by glucose have been the subject of recent study. The carbohydrate response element is a transcription factor identified as mediating glucose-induced Txnip in islet cells.[39] More recently, the same group has extended their studies to suggest that the carbohydrate response element binds to the Txnip promoter and interacts with p300 with subsequent induction of H4 acetylation. USF2 has also been shown to bind to the Txnip promoter and modulate Txnip promoter activity. However, binding of USF2 to the Txnip promoter is not increased by glucose,

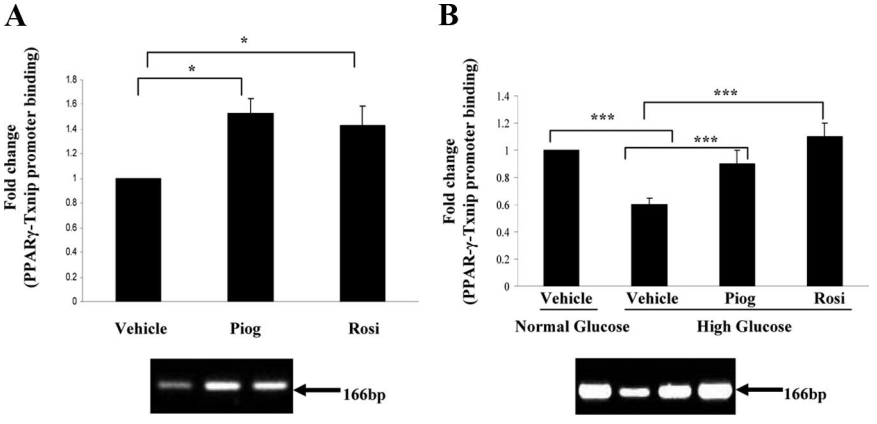

Figure 6. PPAR-γ-Txnip promoter binding activity. HK-2 cells were exposed to vehicle (DMSO), 2 μmol/L pioglitazone (Piog) or 2 μmol/L rosiglitazone (Rosi) (**A**) with the treatment of normal or high glucose for 72 hours (**B**)**.** PCR products were run on 2% agarose gel and a representative gel was shown below each graph with quantification by densitometry using Quantity One software. Results are means ± SEM and are shown as fold change compared with control. Two independent cell culture preparations were performed. $^{*}P < 0.05$; $^{***}P < 0.0005$.

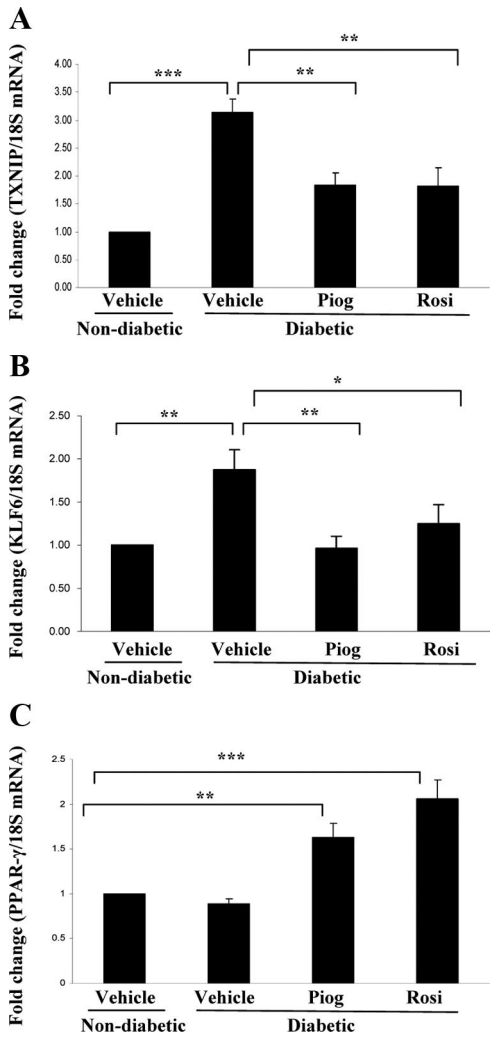

Figure 7. Txnip, KLF6, and PPAR-γ expression in 3-week STZ-induced diabetic rats. Kidney cortex tissues were dissected and snap-frozen. RNA was extracted using TRIzol. Txnip and 18S (**A**), KLF6 (**B**), and PPAR-γ (**C**) real-time PCR was performed after genomic DNA removal. There were six rats in each group. $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0005$. Piog, pioglitazone; Rosi, rosiglitazone.

suggesting that USF2 does not mediate glucose-induced Txnip transcription.[40] The partial inhibition of Txnip mRNA by KLF6 siRNA in high glucose (Figure 1A) is in keeping with both the carbohydrate response element and KLF6 playing a role in high glucose-induced Txnip mRNA expression in HK-2 cells. A recent pilot study suggests that the p38 mitogen-activated protein kinase pathway may up-regulate Txnip expression through forkhead box O1 (FOXO1) by increasing the stability of Txnip mRNA, promoting protein translation in endothelial cells.[41]

Previous studies have demonstrated that overexpression of Txnip increases type IV collagen protein in mesangial cells, suggesting Txnip as a molecular mediator for fibrosis in diabetic nephropathy.[13] Its proapoptotic role has been confirmed in the pancreas because Txnip-deficient mice had increased pancreatic β-cell mass and were protected against STZ-induced diabetes.[9] Targeting Txnip using a sequence-specific Txnip DNAzyme has been shown to enhance cardiomyocyte survival and prevent left ventricular remodeling after myocardial infarction.[42] This study in particular suggests that locally targeting Txnip expression using inhibitors may be a beneficial therapeutic strategy.

The present study has demonstrated that high glucose up-regulates Txnip mRNA and protein expression to a greater extent than would be expected by the observed twofold increase in Txnip promoter activity. Because the magnitude of these increases is consistently observed,[12] it is likely that the relationship of Txnip promoter activity, transcription (mRNA), and translation (protein) is not linear but rather is exponential as discussed previously.[12]

Previous studies have suggested that KLF6 regulates cell proliferation, development, remodeling, and response to injury.[15,17–19] Relevant to the present studies, KLF6 has been shown to be induced in hepatic steatosis rats[18] and to directly transactivate transforming growth factor-β signaling.[43] In recent studies we demonstrated that KLF6 expression is increased in the tubules of diabetic Ren-2 rats undergoing epithelial to mesenchymal transformation, which contributes significantly to the interstitial fibrotic process in diabetic nephropathy. Silencing of KLF6 limits transforming growth factor-β-induced epithelial to mesenchymal transition in HK-2 cells.[21] Clearly this constellation of consequences suggests a key role for KLF6 in the development of diabetes complications. The results of the present study demonstrate that KLF6 up-regulates Txnip expression and silencing of KLF6 significantly attenuates this response. These results suggest that KLF6 may play a broader role in the development of renal complications of diabetic nephropathy, promoting cellular dysfunction beyond the development of the epithelial to mesenchymal transition.

As demonstrated in this study Txnip is not expressed in cells exposed to "normal" glucose concentrations. However, Txnip expression is increased in cells in which PPAR-γ is silenced, hence suggesting a role for PPAR-γ in limiting cellular dysfunction in the basal state. PPAR-γ mRNA expression did not significantly decrease in diabetic rats. This result may be due to the mixed cell population in the kidney cortex or alternatively to the time frame in which the animals were studied. Interestingly, Txnip expression was shown to be increased after treatment with a synthetic PPAR-γ agonist (GW929) in macrophages in a dose-dependent manner. This finding may be explained by the different roles that PPAR-γ plays in different cells and their subpopulations. Consistent with our findings, another study also showed that the macrophage Txnip promoter contains PPAR-γ responsive elements.[44]

The *in vitro* studies further suggest that PPAR-γ agonists do not directly regulate KLF6 expression, and indeed no binding site for PPAR-γ agonists has been identified within the KLF promoter site. However there is at least one KLF6 binding site within the PPAR-γ promoter. This information is consistent with our data showing that PPAR-γ mRNA increased in KLF6-silenced cells, whereas KLF6 expression did not change in cells exposed to PPAR-γ agonists. However, our *in vivo* studies have shown that KLF6 mRNA expression is reduced with thiazolidinedione administration. Hence, the reduction in

KLF6 is likely to be due to a secondary effect of the therapy on metabolic or inflammatory parameters, independent of direct effects on KLF6.

In summary, this study has delineated the roles of KLF6 and PPAR-γ in the regulation of Txnip under high glucose conditions. Early up-regulation of Txnip and KLF6 and reduction of PPAR-γ suggest their important roles in diabetes mellitus.

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