Glucose Up-regulates Thrombospondin 1 Gene Transcription and Transforming Growth Factor-β Activity through Antagonism of cGMP-dependent Protein Kinase Repression via Upstream Stimulatory Factor 2*

Received for publication, February 13, 2004, and in revised form, April 12, 2004
Published, JBC Papers in Press, June 7, 2004, DOI 10.1074/jbc.M401629200

Shuxia Wang‡, Jim Skorczewski, Xu Feng‡, Lin Mei‡, and Joanne E. Murphy-Ullrich‡

From the ‡Department of Pathology, Division of Molecular and Cellular Pathology, The Cell Adhesion and Matrix Research Center and †Department of Neurobiology, University of Alabama at Birmingham, Birmingham, Alabama 35294-0019

Thrombospondin 1 (TSP1) transcription is stimulated by glucose, resulting in increased TGF-β activity and matrix protein synthesis. We previously showed that inducible expression of the catalytic domain of cGMP-dependent protein kinase (PKG) inhibits glucose-regulated TSP1 transcription and transformation growth factor (TGF)-β activity in stably transfected rat mesangial cells (RMCs(tr/cd)). However, the molecular mechanisms by which PKG represses glucose-regulated TSP1 transcription are unknown. Using a luciferase-promoter deletion assay, we now identify a single region of the human TSP1 promoter (∼1172 to ∼578, relative to the transcription start site) that is responsive to glucose. Further characterization of this region identified an 18-bp sequence that specifically binds nuclear proteins from mesangial cells. Moreover, binding is significantly enhanced by high glucose treatment and is reduced by increased PKG activity. Gel mobility shift and supershift assays show that the nuclear proteins binding to the 18-bp sequence are USF1 and -2. USF1 and USF2 bound to the endogenous TSP1 promoter using a chromatin immunoprecipitation assay. Glucose stimulates nuclear USF1 protein accumulation through protein kinase C, p38 MAPK, and extracellular signal-regulated kinase pathways. Increased PKG activity down-regulates USF2 protein levels and its DNA binding activity under high glucose conditions, resulting in inhibition of glucose-induced TSP1 transcription and TGF-β activity. Overexpression of USF2 reversed the inhibitory effect of PKG on glucose-induced TSP1 gene transcription and TGF-β activity. Taken together these data present the first evidence that USF2 mediates glucose-induced TSP1 expression and TSP1-dependent TGF-β bioactivity in mesangial cells, suggesting that USF2 is an important transcriptional regulator of diabetic complications.

It is established that elevated blood glucose levels are a significant risk factor for the development of microvascular complications of diabetes, including diabetic nephropathy (1). Hyperglycemia stimulates an increase in TGF-β activity (2–4), which has been shown to be a major mediator of the fibrotic changes in the pathogenesis of diabetic nephropathy (5, 6).

TGF-β is synthesized and secreted as latent complex (Latent TGF-β). It must be converted to the active state before binding to its receptors and eliciting cellular functions. Latent TGF-β can be activated by a number of factors (7–9). The matricellular protein, thrombospondin1 (TSP1), is a major physiological regulator of TGF-β activation (10–12). TSP1, a 420-kDa homotrimer with individual subunits of ∼145 kDa, is a multifunctional protein whose expression is controlled by many factors (13–16). Our earlier work showed that glucose-stimulated increases in TSP1 expression are responsible for the activation of TGF-β in mesangial cells when exposed to high glucose concentrations (30 mM), which contributes to the accumulation of extracellular matrix proteins (17). Furthermore, high glucose mediates increases in TSP1 expression and TSP1-dependent TGF-β bioactivity through down-modulation of nitric oxide (NO)cGMP-dependent protein kinase (PKG) signaling (18, 19).

PKG is a serine/threonine kinase consisting of an amino-terminal regulatory and a COOH-terminal catalytic domain within one polypeptide chain. Binding of cGMP by the regulatory domain leads to activation of the catalytic domain (20). Although PKG is best known as a regulator of vascular smooth muscle cell (VSMC) contractility, VSMC phenotype, cardiac contractility, intracellular calcium signaling, platelet aggregation, and cytoskeletal reorganization (21–24), PKG also regulates expression of multiple genes such as c-fos, mitogen-activated protein kinase phosphatase 1, gonadotropin-releasing hormones, soluble guanylate cyclase, osteopontin, and TSP1 (18, 19, 22, 24–30). Activation of the cAMP response, cAMP response, activator protein-1 elements, and transcriptional regulatory factor TFII-I are reported to be involved in the PKG-regulated gene transcription (28, 31–33).

In our previous studies we generated stably transfected rat mesangial cells with tetracycline-regulated expression of the catalytic domain of PKG-I to directly regulate the activity of PKG independent of cGMP levels (19). Using these stably...
transfected rat mesangial cells, we showed that expression of the catalytic domain of PKG significantly repressed glucose-induced but not basal TSP1 gene transcription and TSP1-dependent TGFB activation (19). However, the molecular mechanisms by which PKG up-regulates and PKG represses glucose induction of TSP1 transcription are unknown.

In previous work, using the human TSP1 promoter we showed that high glucose concentrations (30 mM) up-regulate the activity of a 2.033-kilobase region of the human TSP1 promoter (18) in a PKG-dependent manner (19) and high glucose-stimulated reporter activity (19). These data suggest that this 2.033-kilobase promoter region contains important regulatory elements required for PKG-mediated repression of glucose-induced TSP1 gene expression. In the present study we investigated the molecular mechanisms by which PKG mediates repression of glucose-induced TSP1 gene transcription in mesangial cells by using a promoter-deletion approach. We now report identification of a region −932 to −915 relative to the transcription initiation site in the TSP1 promoter (18-bp region) that is a binding site for upstream stimulatory factors (USFs), which are critical for glucose-induced TSP1 transcription. Furthermore, we now show that PKG represses glucose stimulation of TSP1 transcription and TGFB activity through down-regulation of USF2 protein and DNA binding, implicating USF2 as a significant transcriptional regulator of diabetic complications.

EXPERIMENTAL PROCEDURES

Chemicals, Reagents, and Antibodies—RPMI 1640 medium with 1-glutamine without glucose was purchased from Invitrogen. Insulin-transferrin-sodium selenite liquid media supplement, minimal essential medium nonessential amino acid solution, and sodium pyruvate of the solution were purchased from Sigma. Synthetic oligonucleotides were purchased from Sigma (Qiagen, Valencia, CA). Wild type and mutant USF consensus oligonucleotides and antibodies used for supershift assay, including USF-1 (sc-229, sc8983), USF-2 (sc-862), c-Myc (sc-674), Max (sc-197), were purchased from Santa Cruz Biotechnology, Inc. (Santa, Cruz, CA). Monoclonal antibody 133, raised against human platelet TSP1 stripped of TGFB activity, was purified by our laboratory in a joint effort with the University of Alabama at Birmingham Hybridxoma Core Facility (34). Luciferase assay reagent, passive lysis buffer, and dual-luciferase reporter assay system were purchased from Promega (Madison, WI). Human recombinant TGFB-1 was purchased from R&D Systems, Inc. (Minneapolis, MN). Glycine sulfate (G418) was obtained from ICN Biomedicals Inc. (Aurora, Ohio). MAPK kinase (MEK) inhibitor PD 98059, p38 MAPK inhibitor SB 202190, c-Jun amino-terminal kinase inhibitor SP600125, and PKC inhibitor bisindolylmaleimide I were purchased from Calbiochem. Dominant negative plasmids and control plasmids for MEK and p38 MAPK were obtained from Dr. Lin Mei from the University of Alabama at Birmingham. Chromatin immunoprecipitation assay kit was obtained from Upstate Biotechnology (Lake Placid, NY).

Cell Culture—Primary rat mesangial cells (RMCs) (passage 3) were a generous gift from Dr. Anne Woods, University of Alabama at Birmingham. As described previously (19), RMCs were stably transfected with pDNA/ATR and pDNA/TGF/onc (a construct containing a catalytic domain of PKG-Iα). These stable transfecnt were labeled as RMCs(tr/cd) and cultured in RPMI 1640 medium supplemented with 20% heat-activated fetal bovine serum, 5 mM t-glutamine, 2 mM t-glutamine, 1% (v/v) nonessential amino acids, 2 mM sodium pyruvate, 0.1 mg/ml transferrin, 5 mg/ml sodium selenite, 0.6 μU, 1.5 μg/ml basicin, and 250 μg/ml zeocin. Serum-free RPMI 1640 media with 5 μg/ml transferrin, 5 mg/ml sodium selenite, and 5 mg t-glutamine were used to quiescent RMCs(tr/cd). 1 μg/ml tetracycline was used to induce the expression of the catalytic domain of PKG to increase PKG activity.

Mink lung epithelial cells (MLECs-clone 32) stably transfected with the TGFB response element of the human plasminogen activator inhibitor-I promoter fused to firefly luciferase reporter gene were a generous gift from Dr. D. B. Rifkin (New York University Medical Center). Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum, t-glutamine, and 200 μg/ml G418.

TSP1 Promoter-Reporter Constructs—A luciferase reporter plasmid containing the −2033 to +750 region of the human TSP1 gene promoter was generously provided by Dr. Paul Bornstein (University of Washington) and used as a template. Eight deletion mutants of TSP1 (−1172), TSP1 (−1112), TSP1 (−1052), TSP1 (−992), TSP1 (−932), TSP1 (−878), TSP1 (−548), and TSP1 (−330) were prepared using forward primers derived from different 5’ positions (the numbers in parentheses contain KpnI site) of the human TSP1 promoter and the same reverse primer (−5′-gctagctgtagcaggaagcacaagag-3′), containing Nhel site. The PCR was performed using TSP1 (−2053−750) as a template and high fidelity Pfu polymerase from Invitrogen. PCR products were digested with KpnI and Nhel and subcloned into a luciferase plasmid PGL 3-Enhancer vector (Promega), and their sequences were confirmed by sequencing.

Transfection and Luciferase Assay—RMCs(tr/cd) were seeded into 6-well plates at a density of 105 cells/ml for 1 day and made quiescent in serum-free RPMI 1640 media with or without tetracycline (1 μg/ml) for 2 days. Then cells were transiently transfected using Effectene transfection reagent (Qiagen) with (1 μg) TSP1 promoter luciferase reporter plasmid. For co-transfection experiments, cells were transiently transfected with 1 μg of TSP1 promoter luciferase reporter plasmid as well as different amounts of USF2 expression vector or empty vector (pShs) (generous gifts from Dr. Michele Sawadogo, the University of Texas, MD Anderson Cancer Center). PRL-SV40 (0.02 μg) (Promega) was used as an internal control. Transfected cells were treated with normal (5 mM) or high (30 mM) glucose for 1 day, and the luciferase activities were assayed using the dual-luciferase assay kit (Promega) according to the manufacturer’s directions.

Nuclear Extract Preparation—RMCs(tr/cd) were cultured in tissue culture dishes with growth media (RPMI 1640 media containing 20% fetal bovine serum). After reaching 80–85% confluence, cells were made quiescent by changing into serum-free RPMI 1640 media for 2 days in the presence or absence of 1 μM tetracycline (to induce the expression of catalytic domain of PKG resulting in increased PKG activity) (19) and then cultured in serum-free RPMI 1640 media with 5 or 30 mM glucose. After 24 h, cells were washed with cold phosphate-buffered saline (pH 7.4), scraped off the dishes, and spun down. Then nuclear extracts were prepared as described previously (35). Briefly, cell pellets were resuspended in 500 μl of hypotonic lysis buffer (10 mM Hepes-KOH (pH 7.9), 10 mM KCl, 1 mM MgCl2, 0.5 mM dithiothreitol, 150 μM phenylmethylsulfonyl fluoride, 5 μg/ml pepstatin, and 5 μg/ml leupeptin). The nuclei were incubated with the extraction buffer on ice for 30 min and spun down. The supernatant (nuclear extract) was stored at −80 °C. Protein concentration of nuclear extracts was determined using the Bio-Rad protein assay kit.

Chromatin Immunoprecipitation Assay—To detect the in situ asso-
cification of nuclear proteins with the TSP1 promoter, the chromatin immunoprecipitation assay was conducted as described by Upstate Biotechnology with some modifications. In brief, primary mesangial cells (p3-6) or stably transfected mesangial cells (RMCs(tr/cd)) were cultured in a 10-cm culture dish. After being quiescent in serum-free RPMI 1640 media for 2 days, cells were treated with normal glucose (5 mM) or high glucose (30 mM) for 24 h. After treatment, protein-DNA complexes were fixed by 1% formaldehyde in phosphate-buffered saline. The fixed cells were washed and lysed in SDS lysis buffer with protease inhibitors and sonicated on ice. After centrifugation at 500 x g for 1 min, one portion of the precleared supernatant was used as DNA input control, and the remaining supernatant was subdivided into aliquots and then incubated overnight at 4°C with nonimmune rabbit serum, anti-sigelolin (6 IgG, Santa Cruz) or IgG antibodies to USF1 or USF2. The immunoprecipitated complexes of antibody-protein-DNA were collected using a protein A slurry, washed successively with low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 150 mM NaCl), high salt buffer (same as the low-salt buffer but with 500 mM NaCl), LiCl buffer (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl (pH 8.1), and Tris-EDTA (pH 8.0), and then eluted with elution buffer (1% SDS, 100 mM NaHCO3). The cross-linking of protein-DNA complexes was reversed by incubation with 5 M NaCl at 65°C for 4 h, and DNA was digested with 10 mg of proteinase K (Sigma/vml) for 1 h at 45°C. The DNA was then extracted with phenol-chloroform, and the purified DNA pellet was resuspended in H2O and subjected to PCR amplification with the forward primer, 5'-GTCGTCCTGCTGAACTACGG-3' and the reverse primer, 5'-GGTCTCTCTAAATTTGTCTCC-3', which were specifically designed from the TSP1 promoter. The 80-bp PCR products were resolved by 3.5% agarose-ethidium bromide gel electrophoresis, visualized by UV.

Site-directed Mutagenesis—Point mutations were introduced into the USF binding site (−924 to −919) in the TSP1 promoter reporter construct (−2033 to −750) using a QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). The primers used to mutate the USF binding site are 5'-gatcgtggagcagatctgctgattc-3' and 5'-ggagatttgcagaactctctggctccagagtc-3' (purchased from Qiagen). PCR was performed in a 50-μl volume with 10 ng of DNA template, 0.2 M MgCl2 (750), and 125 ng of each of the primers using the following conditions: 95°C for 30 s for 1 cycle and 95°C for 30 s, 55°C for 1 min, and 68°C for 12 min for 18 cycles. The PCR products were treated with DpnI (10 units) for 60 min at 37°C. XLI-Blue supercompetent cells were transformed with DpnI-treated PCR mixtures as described in the instruction manual and plated on ampicillin plates. Plasmids were prepared from individual colonies and sequenced to confirm the correctness of introduced mutations.

**RESULTS**

**Glucose-mediated Increases in TSP1 Promoter Activity in RMCs(tr/cd) Are Supported by TSP1 Promoter Region** (−1172 to −878)—We previously showed that high concentrations of glucose mediate increases in TSP1 expression and TSP1-dependent PKG bioactivity through down-modulation of NO-dependent PKG signaling (18). Moreover, we generated stable transfectants expressing the catalytic domain of PKG in mesangial cells using an inducible expression system (tetracycline-induced gene expression (tet-on)). With the expression of the catalytic domain of PKG to increase PKG activity, glucose-induced TSP1 expression was inhibited at the transcriptional level (19). To identify the promoter elements that regulate TSP1 gene transcription in mesangial cells in response to high glucose concentrations and increased PKG activity, a series of TSP1 promoter-luciferase reporter constructs was generated (Fig. 1A) and transiently transfected into RMCs(tr/cd). The promoter activity was measured by assaying the luciferase activity as described under “Experimental Procedures” and was normalized to Renilla luciferase activity. As shown in Fig. 1B, left panel, the longest construct, TSP (−2033), gave rise to a 2.3-fold increase in TSP1 promoter activity in response to high concentrations of glucose (30 mM). Deletion of a 295-bp region (−1172 to −878) totally abolished high glucose responsiveness, suggesting that this 295-bp region is important for high glucose-induced TSP1 transcription. Increased PKG activity inhibited 30 mM glucose-induced TSP1 promoter activity at basal levels (5 mM glucose) (Fig. 1B, right panel). These data suggest that the region between −1172 and −878 in the TSP1 promoter is involved in PKG-mediated repression of TSP1 transcription under high glucose conditions.

**Localization of a 55-bp TSP1 Promoter Region (−932 to −878) That Binds glucose-induced Nuclear Proteins and Mediates the Repression of TSP1 Transcription by Increased PKG under High Glucose Conditions**—To characterize the cis-acting elements in the 295-bp region (−1172 to −878), first we determined whether the 295-bp region binds any nuclear proteins in response to high glucose. Five overlapping oligonucleotides (Oligo I, II, III, IV, and V) spanning the entire 295-bp TSP1 promoter region were synthesized (Fig. 2A), labeled, and used as probes to perform EMSAs with nuclear extracts from normal or high glucose-treated stably transfected mesangial cells (RMCs(tr/cd)) in the absence or presence of tetracycline (Fig. 2B). Only oligo V gave rise to a major band in the EMSA (lane 1). This band was significantly enhanced by high glucose treatment (lane 2). Increased PKG activity diminished this protein-DNA complex (lane 4), suggesting that oligo V contains cis-acting elements involved in PKG-mediated repression of glucose-induced TSP1 transcription. The specificity of this protein-DNA complex was confirmed in the competition assays using cold oligo V as the competitor in the EMSA to abrogate the formation of this complex (Fig. 2C). To rule out the possibility that the stable transfection of mesangial cells alters cell regulation, primary cultured mesangial cells were also used in the above EMSA assay to confirm the association of oligo V with glucose-induced nuclear proteins. Early passage primary mesangial cells (p3-p6) were treated with normal or high glucose media for 24 h, and then nuclear proteins were extracted, and EMSA were performed. As shown in Fig. 2D, oligo V also specifically gave rise a major band in EMSA, which was significantly enhanced by high glucose (30 mM) treatment of primary mesangial cells.

To determine whether the 55-bp TSP1 promoter region (−932 to −878) mediates the repression of TSP1 transcription by increased PKG activity under high glucose conditions, four additional deletion mutants of TSP1 promoter: TSP (−1112), TSP (−1052), TSP (−992), and TSP (−932) were generated (Fig. 3A). These constructs were transiently transfected into mesangial cells, and promoter activity was evaluated by quantifying the luciferase activity (Fig. 3B). Consistent with the EMSA results (Fig. 2B), transfection studies with these mutants indicated that the 55-bp region from −932 to −878 is necessary for glucose-induced TSP1 transcription and that it is also important for PKG repression of TSP1 transcription.

**Identification of an 18-bp Sequence of the TSP1 Promoter That Specifically Binds Glucose-induced USF1 and USF2**—To identify the nuclear proteins that bind to oligo V (55-bp region of TSP1 promoter −932 to −878), we performed a computer analysis using the GCG program, which revealed that this 55
bp contains putative binding sequences for several transcription factors, including NF-1, c-Jun, USF, heat shock transcription factor, C/EBP, and E12. To specifically identify which transcription factors might be important for glucose regulation of TSP1 transcription, we performed competition assays to identify the specific nuclear protein binding sequence within this 55-bp region. Four oligonucleotides (A, B, C, and D) derived from the 55-bp oligo V were synthesized and used as competitors in the EMSA. As shown in Fig. 4B, only oligo A competed efficiently for the nuclear protein binding, suggesting that an 18-bp sequence within the 55-bp region specifically binds the nuclear proteins induced by high glucose concentrations.

The 18-bp sequence contains a CAGATG motif at −924 to −919, which resembles the CANNTG motif of classical E-box cognates and binds both Myc family members (36) and USF proteins (37, 38). This suggests that the nuclear proteins binding to the 18-bp sequence might be members of the Myc family or USF proteins. Thus, we immunologically probed the protein-DNA complex with antibodies against c-Myc, c-Max, USF-1, and USF-2 in gel supershift assays as described under “Experimental Procedures.” For these experiments, gel shifts were carried out with radiolabeled duplex oligo V (55 bp). As shown in Fig. 5A, only anti-USF1 and -USF2 antibodies supershifted the band (lanes 5, 6, 11, and 12), indicating that the nuclear proteins binding to the 18-bp sequence are USF1 and USF2, possibly as heterodimers. Competition studies showed that unlabeled USF oligonucleotide efficiently competes for USF binding, whereas mutant USF oligonucleotide failed to compete (Fig. 5B). These data strongly suggest that the 18-bp sequence in the TSP1 promoter has a USF binding site.

Having demonstrated the binding of transcription factors USF1 or USF2 to the TSP1 promoter in vitro, we further analyzed the in vivo binding of USF to TSP1 promoter by the chromatin immunoprecipitation assay. Primary rat mesangial cells or the stable mesangial cell line (RMC(tr/cd)) was cultured and treated with normal (5 mM) or high glucose (30 mM) for 24 h. After treatment, formaldehyde was added to cross-link the DNA-protein complexes in vivo. After sonication, immunoprecipitation was performed with non-immune IgG. These data show that the association of USF1 or USF2 to the TSP1 promoter in vitro was enhanced by high glucose treatment, and increased PKG activity diminished this protein-DNA association.

The 18-bp USF Binding Sequence Is Functionally Involved in Glucose-induced TSP1 Transcription—To determine whether the 18-bp USF binding sequence is functionally involved in PKG-mediated repression of glucose-induced TSP1 transcription, we introduced point mutations in the 18-bp USF binding site. In the CAGATG motif, TG was converted to GA (Fig. 7A). Competition assays showed that mutant oligo V failed to compet...
pete for USF binding (Fig. 7A), indicating that CAGATG in the 18-bp sequence is a USF binding site. The same mutation was introduced into the TSP-luciferase reporter construct to generate a reporter deficient in the USF binding site (mTSP1). mTSP1 was transiently transfected into RMCs(tr/cd). As expected, high glucose (30 mM) significantly induced TSP1 activity, which was inhibited by increased PKG activity (Fig. 7B). In contrast, high glucose concentrations failed to induce the reporter activity in the promoter construct with the mutated USF binding site. PKG activity did not affect activity of the mutant promoter.

USF2 Protein Levels Are Regulated in Mesangial Cells by Glucose and PKG—We showed that increased PKG activity significantly diminished glucose-induced USF binding (Fig. 2B). To identify whether these changes in USF binding activity reflect changes in the nuclear accumulation of USF1 and/or USF2 protein levels, we performed immunoblot analyses of extracts prepared from RMCs(tr/cd) treated with either normal or high glucose concentrations in the absence or presence of tetracycline as described under “Experimental Procedures.” As
FIG. 3. The 55-bp TSP1 promoter region (−932 to −878) corresponding to oligo V mediates the repression of TSP1 transcription by increased PKG under high glucose conditions. A, 4 deletion mutants of TSP1 promoter, TSP (−1112), TSP (−1052), TSP (−992), and TSP (−932), were constructed by PCR as described under “Experimental Procedures”. B, RMCs(tr/cd) were transfected with the deletion mutants TSP (−1112), TSP (−1052), TSP (−992), and TSP (−932) as described in the legend to Fig. 1B. The experiments were repeated four times, and the representative result is shown. Data are represented as mean of three replicates ± S.D. RLU, relative light units. NG, normal glucose; HG, high glucose.

FIG. 4. Identification of an 18-bp sequence in oligo V (TSP1 promoter region −932 to −878) binding the nuclear proteins using competition assay. A, the diagram of 4 overlapping oligos (A, B, C, D) spanning the 55-bp oligo V was shown. B, oligo V was labeled and used as a probe to perform EMSA with nuclear extract (N.E.) from RMCs(tr/cd) treated with high glucose media for 1 day in the presence or absence of tetracycline (Tet). Excess cold oligos (A–D) were added as competitors (Comp.). Only oligo A (TSP1 promoter region −932 to −915) competes for the binding of oligo V to glucose-induced nuclear proteins. The experiments were repeated three times, and the representative result is shown. NG, normal glucose; HG, high glucose.
shown in Fig. 8, treatment of RMCs(tr/cd) with high glucose up-regulated USF2 (panel B) but not USF1 (panel A) protein accumulation. Moreover, increased PKG activity reduced glucose-induced USF2 to basal levels (5 mM glucose treatment) (panel B in Fig. 8), suggesting that regulation of TSP1 promoter USF binding activity in mesangial cells by high glucose and PKG reflects in part the regulation of USF2 protein accumulation.

In mesangial cells, high glucose environments activate mitogen-activated protein kinase (MAPK) pathways including extracellular signal-regulated kinase, p38 MAPK and c-Jun amino-terminal kinase (39–42). To determine whether glucose signaling through MAPK pathways is involved in USF2 expression, RMCs(tr/cd) were incubated with the MEK inhibitor PD98059, the p38 MAPK inhibitor SB202190, dominant negative MEK, and p38 MAPK plasmids or the c-Jun amino-terminal kinase inhibitor SP600125 in the presence of tetracycline (Tet). Then cells were treated with high glucose (HG) media for 1 day, and nuclear proteins were extracted as described under “Experimental Procedures.” Oligo V was labeled and used as the probe. Supershift assays were performed in the presence of the indicated antibodies and control IgG (3 μg each). Anti-USF1 and USF2 antibodies disrupt and supershift the DNA-protein complex. The blots shown are the representative of three separate experiments. N.E., nuclear extracts. B, competition assays. EMSA was performed using the labeled oligo V and nuclear extracts from RMCs(tr/cd) treated with high glucose media for 1 day in the presence or absence of tetracycline. Excess cold USF consensus oligonucleotide or USF mutant oligonucleotide (mUSF) was added as a competitor. Only USF consensus oligonucleotide competes for the binding of oligo V to glucose-induced nuclear proteins. These experiments were repeated four times, and the representative result is shown.
under normal or high glucose conditions in the absence or presence of tetracycline (data not shown). These data suggest that extracellular signal-regulated kinase and p38 MAPK, but not the c-Jun amino-terminal kinase pathways are involved in glucose-mediated up-regulation of nuclear USF2 levels. However, these pathways do not appear to be important for PKG mediated down-regulation of nuclear USF2 levels.

Glucose-induced activation of the MAPK pathway can be mediated by increased PKC activity in mesangial cells (43, 44). Moreover, glucose-induced PKC activation has been shown to up-regulate TSP1 expression (45). To address the role of PKC in the regulation of high glucose-induced USF2 expression, the PKC inhibitor (bisindolylmaleimide I (Bis)) was used. In preliminary dose-response experiments, 100 nM Bis was found to be an optimal concentration (data not shown). As shown in Fig. 8F, without tetracycline induction Bis (100 nM) compound inhibited 30 mM glucose-mediated USF2 up-regulation. However, with tetracycline induction of PKC activity, Bis compound did not affect USF2 protein levels under normal or high glucose conditions. This result suggests that PKC activity is involved in glucose-induced USF2 expression.

Overexpression of USF2 Reverses PKG-mediated Repression of Glucose-induced TSP1 Expression (Promoter Activity and Protein Levels) and TGF-β Bioactivity—To examine the effects of USF2 on TSP1 promoter activity and TGF-β bioactivity, we transiently co-transfected the expression vector for USF2 with TSP1-luciferase reporter construct (TSP1/H11002). 24 h after transfection, conditioned media were collected to analyze TSP1 protein levels and measure TGF-β bioactivity (Fig. 9C) under high glucose conditions in a concentration-dependent manner in cells treated with tetracycline to induce PKG activity. Similarly, the PKG-mediated decrease in active TGF-β was reversed by the overexpression of USF2 protein (Fig. 9C). However, total TGF-β production was not altered by the overexpression of USF2 protein in mesangial cells (Fig. 9D). Taken together, these data suggest that decreased protein levels and DNA binding activity of USF2 mediates the inhibitory effect of PKG on glucose-induced TSP1 gene expression and TSP1-dependent TGF-β activation in mesangial cells.

DISCUSSION

Accumulating evidence suggests that TSP1 plays an important role in diabetes and diabetic nephropathy (17, 46–51). Previously we demonstrated that high glucose concentrations (30 mM) mediate increases in TSP1 expression and TSP1-dependent TGF-β bioactivity in glomerular mesangial cells through down-modulation of NO/cGMP-dependent protein kinase signaling (18). Moreover, increased PKG activity repressed glucose-induced TSP1 expression at the level of transcription (19). In this present study, we further investigated the molecular mechanisms by which glucose induces and PKG represses TSP1 gene transcription in glomerular mesangial cells. We identified a glucose-responsive region in TSP1 promoter from –932 bp to –915 bp (18bp), which is also involved in PKG-mediated repression of TSP1 transcription under high glucose conditions. Furthermore, we showed that this 18-bp region regulates TSP1 promoter activity by binding USF1 and USF2. Glucose stimulation of USF2 protein expression through PKC, extracellular signal-regulated kinase, or p38 MAPK pathways leads to increased DNA binding activity and contributes to glucose-induced TSP1 transcription and increased TGF-β bioactivity in our studies. PKG inhibits the effects of glucose on TSP1 expression and TGF-β activation through down-regulation of glucose-induced USF2 protein levels.

USFs belong to the basic helix-loop-helix leucine zipper
(b-HLH-LZ) family of transcription factors (52). USFs (USF1 and USF2) were originally identified by their ability to bind to the adenovirus major late promoter (37). USF1 and USF2 are also related to the Myc family of transcription factors and have a similar polypeptide structure and a similar DNA binding specificity (53, 54). USF1 and USF2 are encoded by two distinct genes in human, rat and mouse, and these proteins have molecular masses of 43 and 44 kDa, respectively (54–57). Structurally, USF1 and USF2 are related with a highly conserved COOH-terminal domain responsible for their dimerization and DNA binding (52, 58, 59). The major USF species present in most tissues and cell lines is the heterodimer of USF1 and USF2. USF1 homodimers are less abundant, and USF2 homodimers are usually quite rare (58–60). Both USF1 and USF2 have been shown to bind to the canonical sequence CANNTG (an E-box motif) as either homo- or heterodimers to regulate gene transcription (55, 59, 61, 62).

Although USF1 and USF2 genes are ubiquitously expressed in mammalian cells, the relative abundance of USF1 and USF2 transcripts and protein levels varies among different cell types (59, 61). It has also been shown that the function of USFs is modulated in a cell-specific manner (62). In liver, Vaulont and co-workers (63) demonstrate the contribution of USF1 and USF2 to hepatic glucose responses in mice possessing homologous disruption of these two transcription factor genes. They show that USF1 and USF2 bind in vitro glucose/carbohydrate response elements of glycolytic and lipogenic genes, such as L-type pyruvate kinase and spot 14 genes, and mediate glucose-induced transcriptional activation of these genes (63). Furthermore, in the liver of USF1-deficient mice, enhanced USF2 expression and an increase in the levels of USF2 homodimers compensated for lack of USF1 in the transcriptional regulation of the glucose response elements of these genes.

**A.** Wild type Oligo V (WT Oligo V)

5′ tct gga gcc aga tgg ttc tgc aaa ttc ccc aaa cag gag tca cgt taa gaa gca c 3′

Mutant Oligo V (MUT Oligo V)

5′ tct gga gcc aga **G**ag ttc tgc aaa ttc ccc aaa cag gag tca cgt taa gaa gca c 3′

**B.** Stable mesangial cell line (RMCs(tr/cd))

![Graph showing luciferase activity](image)

**FIG. 7.** Mutation of USF binding site-reduced glucose induced TSP1 promoter activity. A, the competition assay was performed using labeled oligo V as the probe and 100× cold wild type (WT) oligo V or mutant oligo V as the competitor. Nuclear extract (N.E.) for this assay were from RMCs(tr/cd) treated with high glucose media (HG) in the absence or presence of tetracycline (Tet). The results shown are the representative of three separate experiments. B, mutation of TSP (−2033) (mTSP) was generated by introducing two point mutations as shown in Fig. 6A. RMCs(tr/cd) were cultured and made quiescent in serum-free media in the absence or presence of tetracycline for 2 days. Then cells were transiently transfected with wild type TSP1 (−2033) or mutant TSP1 (−2033) as well as internal control pRL-SV40 under normal (NG) or high glucose conditions for 1 day. The promoter activity was quantified by assaying luciferase activity as described under “Experimental Procedures” and normalized to the Renilla luciferase activity of internal control. The experiments were repeated three times, and representative data are shown. Data are the means of three replicates ± S.D. RLU, relative light units.
Fig. 8. Regulation of USF1 and USF2 protein levels in RMCs(tr/cd) by glucose and PKG. RMCs(tr/cd) were cultured and made quiescent in serum-free media with 5 mM glucose in the presence or absence of tetracycline (Tet) for 2 days. Then cells were treated with PD 98059 (10 μM), SB 202190 (10 μM), or Bis (100 nM) or transfected with dominant negative plasmids for MEK or p38 MAPK (DN-MEK, DN-p38), and control plasmids (5 μg each) in normal (NG) or high glucose (HG) media for 1 day. Nuclear extracts were prepared, and USF protein levels were assessed by immunoblotting as described under “Experimental Procedures.” Actin was used as the internal control. The data shown are representative of three separate experiments. Relative USF levels were determined by scanning densitometry of immunoblots. Results are the mean of three experiments ± S.D. *, p < 0.05 for 30 mM glucose versus 5 mM glucose; #, p < 0.05 for 30 mM glucose with tetracycline versus without tetracycline; †, p < 0.05 for 30 mM glucose with PD98059 or SB202190 compounds without tetracycline versus 30 mM glucose without PD98059 or SB202190 compounds without tetracycline. RLU, relative light units.
response of L-type pyruvate kinase and spot 14 genes to glucose. However, in USF2/H11002/H11002/H11002 mice, an impaired glucose responsiveness was observed (64), suggesting that USF2 is essential for transcriptional responses of liver genes to glucose. Our present work shows for the first time that glucose up-regulates USF2 protein accumulation in glomerular mesangial cells and enhances protein-DNA interactions on the TSP1 promoter, resulting in increased TSP1 protein levels and TSP1-dependent TGF-β activation. Furthermore, when the PKC, extracellular signal-regulated kinase, or p38 MAPK pathways are inhibited, glucose-induced USF2 expression is down-regulated, suggesting that the PKC, extracellular signal-regulated kinase, and p38 MAPK pathways are important for glucose-mediated stimulation of USF2 expression. In contrast to USF2, we failed to observe glucose stimulation of USF1 protein accumulation. This differs from the results of Bidder et al. (65) and Weigert et al. (66).

**Fig. 9.** Overexpression of USF2 protein reverses PKG-mediated repression of glucose-induced TSP1 expression (promoter activity and protein levels) and TGF-β activity. RMCs(tr/cd) were cultured and made quiescent in serum-free media for 2 days in the absence or presence of tetracycline (Tet). Then cells were co-transfected with 1 µg of TSP (~2033) luciferase construct and expression vectors for USF2 in normal (NG) or high glucose (HG) media as indicated for 1 day. Empty expression vector (pSG5) was used as control. As described under “Experimental Procedures,” conditioned media were collected, TSP1 protein levels were assayed by immunoblotting, and TGF-β activity was measured in the PAI1 promoter luciferase assay; cells were harvested, and the promoter activity was quantified by assaying luciferase activity. The experiments were repeated three times, and a representative result is shown. Data are represented as the mean of three replicates ± S.D. *, p < 0.05 for 30 mM glucose versus 5 mM glucose; **, p < 0.05 for high glucose with tetracycline versus NG with tetracycline; #, p < 0.05 for high glucose with tetracycline versus high glucose without tetracycline; ##, p < 0.05 for high glucose + USF versus high glucose + empty vector. RLU, relative light units.
In their studies, USF2 protein levels are down-regulated by increased PKG activity, which contributes to decreased USF binding activity, resulting in repression of TSP1 gene transcription under high glucose conditions. Regulation of USF2 at the level of transcription, mRNA stabilization, translation, protein degradation, or some combination thereof probably contributes to the decreased USF2 protein levels mediated by PKG. We found no evidence for the involvement of PKC, extracellular signal-regulated kinase and p38 MAPK pathways in PKG-mediated down-regulation of USF2, and the mechanisms by which PKG down-regulates USF2 remain to be determined.

Our studies show that overexpression of USF2 reverses PKG-mediated repression of glucose-induced TSP1 expression and TSP1-dependent TGF-β activation. However, total TGF-β production is not altered by the overexpression of USF1 and/or USF2 enhanced TGF-β1 promoter activity in a simian virus 40 (SV40)-transformed mouse mesangial cell line. Experimental conditions such as transfection efficiency, SV40 transformation, or species-specific regulation of USF-mediated TGF-β expression in mesangial cells might contribute to this difference.

In summary, our data provide the first evidence that USF2 mediates glucose stimulation of TSP1 gene transcription and protein expression and TSP1-dependent TGF-β bioactivity in mesangial cells, suggesting that USF2 is an important transcriptional regulator of diabetic nephropathy. Moreover, increased PKG activity down-regulates glucose-induced USF2 protein, resulting in a decrease in protein-DNA activity, contributing to PKG-mediated repression of glucose-induced TSP1 gene expression and TSP1-dependent TGF-β activation.

Acknowledgments—We thank Dr. Paul Bornstein, University of Washington, for the generous gift of TSP1 gene promoter reporter constructs and Dr. Michele Sawadogo, University of Texas MD Anderson Cancer Center for providing USF1 and USF2 expression vectors.

REFERENCES

1. Diabetes Control and Complications Trial Research Group (1993) N. Engl. J. Med. 329, 977–988
2. Ziyadeh, F. N., Sharma, K., Erickson, M., and Wolf, G. G. (1994) J. Clin. Invest. 93, 536–542
3. Wolf, G., Sharma, K., Chen, Y., Erickson, M., and Ziyadeh, F. N. (1992) Kidney Int. 42, 647–656
4. Sharma, K., and Ziyadeh, F. N. (1995) Diabetes 44, 1139–1146
5. Ziyadeh, F. N. (1995) Am. J. Kidney Dis. 22, 736–744
6. Iwano, M., Kubo, A., Nishino, T., Sato, H., Nishio, H., Akiy, Y., Kurioka, H., Fuji, Y., Kanauchi, M., Shiuki, H., and Dohi, K. (1996) Kidney Int. 49, 1120–1126
7. Riikonen, D., Kajimo, S., Abe, M., and Harpel, J. G. (1993) Thromb. Haemostasis 70, 177–179
8. Lyon, R. M., Gentry, L. E., Purchio, A. F., and Moses, H. L. (1990) J. Biol. Chem. 265, 13881–13887
9. Taipale, J., Lohi, J., Seasholtz, T. M., Boss, G. R., and Pilz, R. B. (2002) J. Biol. Chem. 277, 57283–57289
10. Kinoshita, C., Nissio, G., Moldolesi, J., and Clementi, E. (1997) Br. J. Pharmacol. 122, 687–697
11. Tolsma, S. S., Volpert, O. V., Good, D. J., Frazier, W. A., Pelverini, P. J., and Enoch, N. (1993) J. Cell. Biol. 123, 497–511
12. Liu, Y., Shi, Z., Silvera, A., Liu, J., Sawadogo, M., Yang, H., and Feng, X. (2003) J. Biol. Chem. 278, 20603–20611
13. Luscher, B. (2001) Gene (Amst.) 277, 1–14
14. Sawadogo, M. (1998) J. Biol. Chem. 273, 1399–1401
15. Sawadogo, M., Van Dyke, M. W., Gregor, P. D., and Roeder, R. G. (1988) J. Biol. Chem. 263, 1195–1199
16. Tuino, R., Zabel, F., Fantus, I. G., Dlugosz, A., and Whiteside, C. (2002) Am. J. Physiol. Endocrinol. Metab. 282, 161–169
17. Wilmer, W. A., Dixon, C. L., and Hebert, C. (2001) Kidney Int. 60, 858–871
18. Hanesda, M., Araki, S., Togawa, M., Sugimoto, T., Isono, M., and Kikkawa, R. (1997) Diabetes 46, 1327–1332
19. Ingram, A. J., Li, H., Thai, K., Kang, M. J., and Scholey, J. W. (1999) Kidney Int. 56, 1721–1728
20. Hanesda, M., Kikkawa, R., Sugimoto, T., Koya, D., Araki, S., Togawa, M., and Shigeta, Y. (1995) J. Diabetes Complications 9, 248–248
21. Hanesda, M., Koya, D., and Kikkawa, R. (2001) Am. J. Kidney Dis. 38, Suppl. 1, S178–S181
22. Tada, H., Kubok, N., Nomura, K., and Inokuchi, T. (2001) J. Diabetes Complications 15, 193–197
23. Bayraktar, M., Dundar, S., Kirazli, A., and Telelter, P. (1994) J. Int. Med. Res. 22, 90–94
24. Hida, K., Wada, J., Zhang, H., Hiragushi, K., Tsuchiyama, Y., Shikata, K., and Makino, H. (2006) J. Lipid Res. 47, 1615–1622
25. Stefanou, O. I., Kroukaves, I. G., Shi, X., Zhu, Z., Forodi, F., Penn, F. M., Tso, E. J., and Pfeiffer, G. (2016) Proc. Natl. Acad. Sci. U. S. A. 107, 3209–3215
26. Holmes, D. I., Abdel Wahab, N., and Mason, R. M. (1997) Biochem. Biophys. Res. Commun. 238, 179–184
27. Murphy, M., Godson, C., Cannon, S., Kato, S., Mackenzie, H. S., Martin, F., and Brady, H. R. (1999) J. Biol. Chem. 274, 5830–5834
28. McGregor, B., Colon, S., Mutin, M., Chignier, E., Zech, P., and McGregor, J. (1994) Am. J. Pathol. 144, 1281–1287
29. Litwack, T. D., and Ivan, G. I. (1995) Protein Profile 2, 621–702
30. Kadesch, T. (1996) Cell Growth Diff. 4, 49–55
31. Henris, A. A., Arens, A. M., Mattei, M. G., Kahn, A., and Raymondj, M. (1995) Genomics 36, 45–45
32. Henris, A. A., Vaulont, S., Raymondj, M., and Kahn, A. (1996) Mamm. Genome 7, 803–809
33. Aperlo, C., Boulakos, K. E., Sage, J., Cuzin, F., and Pognonec, P. (1996) Genomics 37, 337–344
34. Lin, Q., Luo, X., and Sawadogo, M. (1994) J. Biol. Chem. 269, 23894–23903
35. Srinith, M., Lin, Q., Matty, T., and Sawadogo, M. (1994) Nucleic Acids Res. 22, 427–433
36. Volett, B., Lefrancosis-Martinez, A. M., Henris, A., Kahn, A., Raymondj, M., and Martinez, A. A. (1996) J. Biol. Chem. 271, 1405–1415
37. Srinith, M., Lin, Q., Deng, J. M., Behringer, R. R., and Sawadogo, M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 3758–3763
38. Srinith, M., Walker, B., Liu, Q., and Srinith, M. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 8880–8885
39. Varambally, S., and Srinith, M. (1992) Gene Expr. 2, 45–51
40. Qyang, Y., Luo, X., Li, T., Ismaili, P. M., Krylov, D., Vinson, C., and Sawadogo, M. (1999) Mol. Cell. Biol. 19, 1508–1517
41. Vallet, V., S., Casado, M., Henris, A., A., Bucchini, D., Raymondj, M., Kahn, A., and Vaulont, S. (1998) J. Biol. Chem. 273, 20175–20179
42. Vallet, V., S., Henris, A., Casado, M., Raymondj, M., and Martinez, A. M. (1997) J. Biol. Chem. 272, 21914–21919
43. Bidder, M., Shin, J. S., Charleston-Kachang, N., Loewy, A. P., Semenkovich, C. F., and Trower, D. A. (2002) J. Biol. Chem. 277, 44485–44496
44. Weigert, C., Brodbek, K., Sawadogo, M., Haring, H. U., and Schlesier, E. D. (2004) J. Biol. Chem. 279, 15908–15915
Glucose Up-regulates Thrombospondin 1 Gene Transcription and Transforming Growth Factor-β Activity through Antagonism of cGMP-dependent Protein Kinase Repression via Upstream Stimulatory Factor 2

Shuxia Wang, Jim Skorczewski, Xu Feng, Lin Mei and Joanne E. Murphy-Ullrich

J. Biol. Chem. 2004, 279:34311-34322.
doi: 10.1074/jbc.M401629200 originally published online June 7, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M401629200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 66 references, 31 of which can be accessed free at http://www.jbc.org/content/279/33/34311.full.html#ref-list-1