Upstream Stimulatory Factor (USF) Proteins Induce Human TGF-β1 Gene Activation via the Glucose-response Element −1013/−1002 in Mesangial Cells

UP-REGULATION OF USF ACTIVITY BY THE HEXOSAMINE BIOSYNTHETIC PATHWAY*

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The hyperglycemia-enhanced flux through the hexosamine biosynthetic pathway (HBP) has been implicated in the up-regulated gene expression of transforming growth factor-β1 (TGF-β1) in mesangial cells, thus leading to mesangial matrix expansion and diabetic glomerulosclerosis. Since the −1013 to −1002 region of the TGF-β1 promoter shows high homology to glucose-response elements (GIRE) formerly described in genes involved in glucose metabolism, we studied the function of the GIRE in the high glucose-induced TGF-β1 gene activation in mesangial cells. We found that high glucose concentrations enhanced the nuclear amount of upstream stimulatory factors (USF) and their binding to the sequence. Fusion of the GIRE to the thymidine kinase promoter resulted in glucose responsiveness of this promoter construct. Overexpression of either USF-1 or USF-2 increased TGF-β1 promoter activity 2-fold, which was prevented by mutation or deletion of the GIRE. The high glucose-induced activation of the GIRE is mediated by the HBP; increased flux through the HBP induced by high glucose concentrations, by glutamine, or by over-expression of the rate-limiting enzyme glutamine:fructose-6-phosphate aminotransferase (GFAT) particularly activated USF-2 expression. GFAT-overexpressing cells showed higher USF binding activity to the GIRE and enhanced promoter activation via the GIRE. Increasing O-GlcNAc modification of proteins by streptozotocin, thereby mimicking HBP activation, also resulted in increased mRNA and nuclear protein levels of USF-2, leading to enhanced DNA binding activity to the GIRE. USF proteins themselves were not found to be O-GlcNAc-modified. Thus, we have provided evidence for a new molecular mechanism linking high glucose-enhanced HBP activity with increased nuclear USF protein levels and DNA binding activity and with up-regulated TGF-β1 promoter activity.

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The adverse effects of hyperglycemia in human and experimental diabetic nephropathy have been linked to the enhanced expression and bioactivity of the prosclerotic cytokine transforming growth factor-β1 (TGF-β1)* (1–3). The hyperglycemia-induced TGF-β1 stimulates the production of extracellular matrix proteins in mesangial cells and other renal cells (4–7), thus leading to the thickening of glomerular and tubulobasement membranes and the progressive expansion of the glomerular mesangium and the tubulointerstitium (8, 9). Increased renal expression of TGF-β1 has been found in experimental and human diabetes (2, 3) and has also been demonstrated in high glucose-treated renal mesangial and tubular cells (5, 6). The molecular mechanism of up-regulated human TGF-β1 gene expression involves protein kinase C- and p38 MAPK-dependent pathways (10–12) leading to AP-1 activation (13, 14) and subsequently enhanced TGF-β1 promoter activity via two adjacent AP-1 binding sites located at −418/−412 and −371/−363, respectively (13).

Moreover, increased synthesis of amino sugars through the hexosamine biosynthetic pathway (HBP) has also been implicated in hyperglycemia-induced TGF-β1 synthesis (15, 16). Inhibition of the hexosamine pathway prevented the high glucose-induced TGF-β1 synthesis and bioactivity and the enhanced expression of matrix proteins in mesangial cells (15). Furthermore, glucosamine, which promotes flux through the hexosamine pathway distal of the rate-limiting enzyme glutamine fructose-6-phosphate aminotransferase (GFAT) (17), activates the expression of TGF-β1 and several matrix proteins (15). These effects were also observed by overexpression of GFAT in NIH 3T3 fibroblasts (18) and mesangial cells (19) in physiological glucose concentrations. The HBP has been implicated in increased promoter activity of genes under high glucose conditions (20–23). The product glucosamine-6-phosphate (GlcN-6-P) is very rapidly further converted and activated to UDP-GlcNAc, the substrate of an O-GlcNAc transferase that modifies serine and threonine residues of cytosolic and nuclear proteins with a single monosaccharide O-GlcNAc group (20). This O-GlcNAc modification has been proven to be a regulator of protein activities, e.g. of the transcription factor Sp1 (16, 22, 24).

* The abbreviations used are: TGF-β1, transforming growth factor-β1; AP-1, activator protein-1; EMSA, electrophoretic mobility shift assay; GFAT, glutamine-fructose-6-phosphate aminotransferase; GlcN, glucosamine; GIRE, glucose-response element; HBP, hexosamine biosynthetic pathway; MAPK, mitogen-activated protein kinase; O-GlcNAc, O-linked N-acetylglucosamine; RT, reverse transcription; Sp1, stimulatory protein 1; STZ, streptozotocin; TK, thymidine kinase; USF, upstream stimulatory factor.

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24). However, the identification of the HBP-response elements in the TGF-β1 promoter region and of the involved transcription factors remains open.

Examination of the human TGF-β1 promoter region by computer analysis for transcription factor binding sites revealed that the region –1013 to –1002 (CAGTCGCGCCGCC) shows high homology to consensus motifs in the promoters of the liver pyruvate kinase (L-PK), the S14, and the fatty acid synthase gene, which refer to the glucose responsiveness of these genes, and are therefore called carbohydrate or glucose-responsive elements (GIRE) (25, 26). Among the proteins that were reported to possess GIRE DNA binding activity are members of the basic helix-loop-helix leucine zipper family of transcription factors, the upstream stimulatory factors (USF), which bind to the helix-loop-helix leucine zipper family of transcription factors, to possess GlRE DNA binding activity are members of the basic

Materials and Methods

Materials and Plasmids—Mouse mesangial cell line SV40 (27) was obtained from ATCC (Manassas, VA). Oligonucleotides were synthesized by Invitrogen. Cell culture media, supplements, Ultroser, and fetal calf serum were purchased from Invitrogen; minocyclin was from Pan Systems (Aidenbach, Germany); first strand cDNA synthesis kit, Light Cycler system, FuGENE 6, Klenow enzyme, and poly[d(I-C)] were obtained from Roche Applied Science; pG3Lb, pRL-TK, and the Dual luciferase assay system were from Promega (Madison, WI); protein assay reagent was from Bio-Rad; india ink was from Pelikan (Hannover, Germany); [α-32P]dATP was from Hartmann (Braunschweig, Germany); streptozotocin (STZ) predominantly activated the expression of the TGF-β1 gene expression. We found enhanced binding of an USF-1 and -2 heterodimer to the GIRE in nuclear extracts obtained from high glucose-stimulated mesangial cells. Expression of either USF-1 or USF-2 induced TGF-β1 promoter activity 2-fold with no additive effect in the presence of both USF proteins. Mutation or deletion of the GIRE prevented the USF-induced promoter activation almost completely. Increased flux through the HBP stimulated by high glucose or glutamine, by overexpression of GFAT, or enhancement of O-glycosylation by streptozotocin (SZT) predominantly activated the expression of USF-2, although USF proteins were not a direct target of O-GlcNAc modification. Thus, activation of the HBP, via elevated O-glycosylation activity, enhanced nuclear USF protein levels, and increased binding of USF proteins to the GIRE, up-regulates TGF-β1 promoter activity.

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Results

High Glucose Stimulates Binding of Nuclear Proteins USF-1 and USF-2 to the GIRE—To characterize the TGF-β1 promoter region −1013/-1002 as a glucose-response element, the binding of nuclear proteins from normal and high glucose-treated porcine mesangial cells to this region was studied by electrophoretic mobility shift assay (EMSA). Incubation with 30 mM high glucose for 15 h induced the DNA binding activity to the −1013/-1002-containing sequence, and this effect was enhanced after 40 h of high glucose stimulation and persisted for at least 72 h (Fig. 1A). The specificity of the binding was tested by using 30-fold molar excess of unlabeled GIRE oligonucleotide, which prevented the DNA binding completely, whereas 30-fold molar excess of the mutated GIRE does not (Fig. 1B, lane 2 and 3). Moreover, no binding to the radiolabeled mutated GIRE was detected (Fig. 1B, lane 4). To identify the transcription factors that bind to the TGF-β1 GIRE sequence, antibodies against USF-1 and USF-2 were added to the binding reaction. With either antibody, the GIRE-protein complex was completely supershifted, leading to distinct new bands with lower mobility (Fig. 1C, lanes 3 and 4). In contrast, addition of an anti-Sp1 antibody did not affect the mobility of the GIRE-protein complex (Fig. 1C, lane 2). Thus, USF-1 and USF-2 are identified as major components and proved to be essential for the nuclear DNA binding activity to the GIRE.

High Glucose Stimulates Promoter Activation via the GIRE—The functional activity of the TGF-β1 promoter GIRE was studied as the ability of this promoter region to confer high glucose responsiveness to a different promoter. Fusion of the TGF-β1 promoter region −1022/-993 to the thymidine kinase promoter (Fig. 2A), which alone is not activated by high glucose concentrations in porcine mesangial cells (Fig. 2B, TK), led to a 1.5-fold induction of GIRE-TK promoter activity after 24 h of high glucose stimulation (Fig. 2B). Thus, the TGF-β1 promoter region −1022/-993 is a functionally active and independent GIRE.

Expression of USF Proteins Enhances TGF-β1 Promoter Activity—To investigate whether high glucose-induced binding of USF to the GIRE can enhance TGF-β1 promoter activity, SV40 mesangial cells were cotransfected with expression vectors for USF-1 and/or USF-2 and TGF-β1 promoter constructs in pGL3basic in 5.5 mM ambient glucose concentrations. In these experiments, we used SV40 mesangial cells since the transfection efficiency obtained with porcine mesangial cells, although suitable to perform luciferase reporter gene assays, is not sufficient to investigate the effect of transfected proteins. In transfected SV40 mesangial cells, expression levels of USF-1 and USF-2 were high as measured by Western blotting with specific antibodies (Fig. 3A), thus leading subsequently to enhanced binding to the GIRE (data not shown). The nuclear amount of the transcription factor c-Jun was essentially unchanged in USF-transfected cells (Fig. 3A). Promoter activity of the co-transfected −1065/+11 TGF-β1 promoter region (Fig. 3B) increased by either enhanced USF-1 or enhanced USF-2 levels 1.5–2.0-fold with no additive effect in the presence of elevated concentrations of both USF proteins (Fig. 3C). Mutation of the GIRE, which abrogates USF protein binding as demonstrated by EMSA (Fig. 1B), clearly reduces the effect of USF overex-
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Fig. 2. Human TGF-β1 promoter region −1022 to −993 is a functional active GIRE. A, pTK contains the herpes simplex virus thymidine kinase promoter fragment of pRL-TK cloned into pGL3b. In pGIRE-TK, the GIRE sequence of the human TGF-β1 promoter (−1022/−993) is cloned into the BglII site of the multiple cloning site. B, porcine mesangial cells were transfected with pGL3b-TK (TK) or with the pGL3bGIRE-TK construct (GIRE-TK) and cultured in ambient 5.5 mM (normal glucose (NG)) or 30 mM glucose (high glucose (HG)) concentrations and harvested after 24 h. Transfection efficiencies were normalized to cotransfected pRL-TK. Luciferase activities of mesangial cells transfected in normal glucose conditions are set as 100%. Data are means ± S.E. of three independent experiments.

Fig. 3. Overexpression of USF proteins increase TGF-β1 promoter activity. SV40 mesangial cells were cotransfected with expression vectors coding for USF-1, -2, or empty expression vector (con) and TGF-β1 promoter constructs in pGL3basic. A, Western blotting of nuclear extracts of transfected cells. Shown are representative immunoblots for USF-1 and USF-2. The blot was also probed for c-Jun, demonstrating equal protein loading. B, construct of the 5′-flanking region of the TGF-β1 gene in pGL3basic, which contains the firefly (Photinus pyralis) luciferase coding region (LUC). Characterization of the AP-1 binding sites A and B has been described recently (13). C, effect of transfection of USF proteins on TGF-β1 promoter region −1065/+11, −1065/+11mut containing mutated GIRE and −453/+11 constructs. Cells were incubated with ambient 5.5 mM glucose, harvested 24 h after transfection, and assayed for luciferase activities. Luciferase activity measured in cells transfected with empty expression vector was defined as 100%. Data are means of at least three independent experiments.

HBP is provided when SV40 mesangial cells were transfected with GFAT expression vector, leading to high expression of GFAT protein as compared with control cells (Fig. 5A). We found an up-regulation of USF-2 mRNA levels in GFAT-overexpressing cells, whereas again, no increase in USF-1 expression was detected (Fig. 5B). Moreover, only in the GFAT-overexpressing cells, glutamine (16 mM) enhanced USF-2, but not USF-1, mRNA expression as compared with corresponding basal levels in cells grown in ambient normal glucose concentrations (1.57 ± 0.11, data not shown). Accordingly, the USF binding to the GIRE is enhanced by GFAT overexpression (Fig. 5C, lane 2 versus lane 1), identified by supershifted bands in the presence of anti-USF antibodies (Fig. 5C, lanes 3 and 4). Subsequently, the promoter activity of the GIRE-TK construct was found to be up-regulated in the GFAT-overexpressing cells (2.5-fold; Fig. 5D), whereas the promoter activities of the TK promoter alone or the construct containing mutated GIRE were comparably low (1.6-fold versus promoter activities found in control cells; Fig. 5D). This residual activation could be explained by HBP-enhanced transcriptional activity of the ubiquitous transcription factor Sp1, thereby affecting TK promoter activity. Thus, activation of USF-2 expression by high glucose mediated by the HBP appears to be the predominant mechanism leading to enhanced TGF-β1 gene activation via GIRE.
Effect of Enhanced Nuclear O-GlcNAc Modification by STZ on USF-2—O-GlcNAc modification of nuclear proteins is a common phenomenon, leading to enhanced activity of transcription factors, for example, by enhanced nuclear translocation or DNA binding activity (20, 22, 24, 33). Since we found that treatment of mesangial cells with STZ resulted in elevated USF-2 mRNA expression (Fig. 4C), we studied the effect of STZ in more detail. First we investigated the effect of STZ on the nuclear amount of USF-2 protein. Incubation of mesangial cells with 5 mM STZ increased the nuclear USF-2 protein levels in a time-dependent manner (Fig. 6A). Similarly, the DNA binding activity of the USF proteins to the GIRE was found to be activated after 6 and 24 h of STZ-treatment (Fig. 6B). In supershift experiments, the intensity of the USF-2-GIRE complex supershifted by the anti-USF-2 antibody was clearly increased after stimulation with STZ (Fig. 6B). Quite unexpectedly, addition of the anti-O-GlcNAc antibody RL-2 did not result in any supershifted bands (Fig. 6B). To demonstrate the effectiveness of the method to identify O-GlcNAc-modified DNA-binding proteins, we studied the DNA binding of the O-GlcNAc-modified transcription factor Sp1 (16, 24, 33) to a Sp1 consensus binding site after preincubation with the RL-2 antibody. In fact, the Sp1-DNA complex is clearly supershifted by the anti-O-GlcNAc antibodies, whereas again, USF binding activity was unaffected (Fig. 6C). To support the finding that the nuclear USF proteins that bind to the GIRE are not O-GlcNAc-modified, we incubated nuclear proteins with β-N-acetylglucosaminidase before EMSA to remove any potential O-GlcNAc groups on USF proteins. This treatment had no effect on the DNA binding activity of USF proteins to the GIRE of the TGF-β1 promoter, whereas the DNA binding of Sp1 to the high affinity Sp1 binding site is completely prevented (Fig. 6D). Thus, it appears unlikely that USF-1 and -2 activities are regulated by direct O-GlcNAc modification and that these proteins are direct targets of O-GlcNAc-transferase.

DISCUSSION

In the present study, we describe a new link of the HBP to enhanced TGF-β1 gene activation via a hitherto unknown mechanism involving USF transcription factors. USF proteins were found to be responsible for high glucose-induced TGF-β1 promoter activation mediated by a GIRE sequence located at −1013 to −1002 of the TGF-β1 promoter: (i) high glucose concentrations activate binding of a USF-1/USF-2 heterodimer to the GIRE, (ii) the nuclear amount of both proteins is elevated in high glucose-treated cells, (iii) high glucose concentrations induce USF-2 mRNA levels, and (iv) overexpression of USF-1 and USF-2 activates TGF-β1 promoter activity via the GIRE. USF-1 and USF-2 have been shown to be involved as regulators of a high number of genes in numerous cellular processes. Gene knock-out mice and cell culture studies indicate that USFs are important for a kinetically normal activation of diet-dependent genes by glucose in the liver (26, 34–37). However, several studies revealed that these transcription factors are not capable by themselves of confering glucose responsiveness to hepatic and beta cell genes (38–40). Moreover, numerous studies do not demonstrate transcriptional activation of USF by glucose (38–40), e.g. there is evidence that USF is not the glucose-responsive factor that stimulates S14 gene and liver pyruvate kinase expression (38, 39). Thus, in the liver and beta cells, USF proteins appear to not be activated by high glucose concentrations.

In a recent report, glucose-induced USF protein accumulation and subsequently enhanced binding to osteopontin promoter sequences and up-regulation of osteopontin transcription have been demonstrated in vascular smooth muscle cells (41). In our study, we provide the first evidence for a mechanism of high glucose-enhanced USF activity and the increases in USF-2 expression: (i) activation of the HBP by high glucose or glutamine or (ii) increased flux through the HBP by elevated glucose metabolism.
levels of the rate-limiting enzyme GFAT or (iii) by stimulation of O-GlcNAc modification by STZ resulted in increased USF-2 expression and elevated binding to the GlRE. The HBP produces UDP-GlcNAc, the substrate of an O-GlcNAc transferase, which modifies serine and threonine residues of cytosolic and nuclear proteins with a single monosaccharide O-GlcNAc group (20). STZ, an analog of GlcNAc, is known to inhibit /H9252-N-acetylglucosaminidase (O-GlcNAcase), an enzyme that cleaves GlcNAc residues from intracellular proteins (32, 42). The resulting increases in O-GlcNAc modification of proteins modulate their function, e.g. the transcription factor Sp1 is activated (16, 22, 24, 43). Thus, the possible mechanism for up-regulation of USF-2 mRNA levels via the HBP is enhanced O-GlcNAc modification of transcription factors responsible for USF-2 gene activation. Of note, the putative promoter region of the human USF-2 gene is GC-rich, suggesting several Sp1 binding sites (44). A further possibility for the elevated nuclear USF-1 and USF-2 protein levels is O-GlcNAc modification of the USF proteins themselves, thereby inducing their shuttle from cytosol to the nucleus. This has been demonstrated for the transcription factor Sp1 (24, 43). However, using O-GlcNAc-specific antibodies, we did not find any evidence for O-glycosylation of nuclear USF proteins. Therefore, we conclude that the effect of an increased flux through the HBP on USF activity is to up-regulate USF-2 expression. The mechanism responsible for the elevated USF-1 protein levels appears to be independent from a transcriptional regulation of USF-1 by the HBP. Moreover, we could not exclude from our data other, e.g. posttranslational, mechanisms leading to high glucose-induced activation of USF proteins.

With the present study, we obtained additional insights in the molecular mechanism of the hyperglycemia-induced up-regulation of TGF-$\beta$1 expression in mesangial cells. In a previous study, we described the involvement of two AP-1 binding sites in the activation of human TGF-$\beta$1 promoter region $\sim$453/ +11 by high glucose (13); here, we found that a glucose-response element homolog sequence located more distal in the human TGF-$\beta$1 promoter is also activated by high glucose.
concentrations. To study the functional relevance of this GlRE sequence of the TGF-β1 promoter, we investigated the activity when fused to a glucose-independent promoter. We chose this experimental approach since studying the high glucose response of the complete TGF-β1 promoter construct revealed a similar, although weaker (1.5-fold, data not shown), promoter activation compared with the previously published 2.0-fold induction of the shorter TGF-β1 promoter construct (13). An explanation for this unexpected result could be that the human TGF-β1 promoter contains silencing elements between nucleotides 731 and 453 (45), which reduce basal promoter activity to 10% (45) and could cover a high glucose effect. Thus, the stimulatory effect of high glucose on the TGF-β1 promoter regions –1065/+11 and –453/+11 appears similar without any additive effect of the high glucose-inducible AP-1 sites and the GlRE. Of note, a similar glucose-response element has been postulated to be involved in the high glucose-mediated up-regulation of the mouse TGF-β1 promoter (46).

In conclusion, we have ascertained that the human TGF-β1 gene contains a functional active GlRE, which is capable of inducing TGF-β1 gene activation in mesangial cells after expo-

Fig. 6. Involvement of O-GlcNAc modification in increased production and DNA binding activity of USF proteins. Porcine mesangial cells were incubated with 5 mM STZ as indicated. A, Western blotting of nuclear extracts. Shown is one representative immunoblot for USF-2 and india ink staining of a representative, strong band visible at 100 kDa to demonstrate equal protein loading. B, EMSAs were performed with nuclear extracts obtained from STZ-treated porcine mesangial cells with GlRE oligonucleotide. For supershift experiments, nuclear extracts were preincubated with anti-USF-2 antibody or anti-O-GlcNAc antibody. Supershifted USF-2-DNA complex is marked by the bracket. Con, unstimulated cells. C, supershift experiment using anti-O-GlcNAc antibody and a high affinity Sp1 binding site or GlRE. Supershifted Sp1-DNA complex is marked by the bracket. Free DNA probe is not shown. D, prior to the protein DNA binding reaction, nuclear extracts were incubated with β-N-acetylglucosaminidase (hex) for 30 min at RT as indicated. Binding of Sp1 or USF proteins is marked by the arrow, and the free DNA probe is not shown.
High Glucose Concentrations Activate USFs

sure to high glucose concentrations. Thereby, we have provided evidence for a new molecular mechanism linking high glucose-enhanced HBP activity with the transcriptional activation of the TGF-β1 gene via USF protein activation.

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