Evidence for a Novel TGF-β1–Independent Mechanism of Fibronectin Production in Mesangial Cells Overexpressing Glucose Transporters

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Recent experimental work indicates that the hyperglycemia-induced increase in mesangial matrix production, which is a hallmark in the development of diabetic nephropathy, is mediated by increased expression of GLUT1. Mesangial cells stably transfected with human GLUT1 mimic the effect of hyperglycemia on the production of the extracellular matrix proteins, particularly fibronectin, when cultured under normoglycemic conditions. Our investigation of the molecular mechanism of this effect has revealed that the enhanced fibronectin production was not mediated by the prosclerotic cytokine transforming growth factor (TGF)-β1. We found markedly increased nuclear content in Jun proteins, leading to enhanced DNA-binding activity of activating protein 1 (AP-1). AP-1 inhibition reduced fibronectin production in a dosage-dependent manner. Moreover, inhibition of classic protein kinase C (PKC) isoforms prevented both the activation of AP-1 and the enhanced fibronectin production. In contrast to mesangial cells exposed to high glucose, no activation of the hexosamine biosynthetic, p38, or extracellular signal–related kinase 1 and 2 mitogen-activated protein kinase pathways nor any increase in TGF-β1 synthesis could be detected, which could be explained by the absence of oxidative stress in cells transfected with the human GLUT1 gene. Our data indicate that increased glucose uptake and metabolism induce PKC-dependent AP-1 activation that is sufficient for enhanced fibronectin production, but not for increased TGF-β1 expression.
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ANG, angiotensin; AP-1, activating protein 1; ATF, activating transcription factor; CRE, cAMP-responsive element; CREB, CRE binding protein; DCFDA; EGF, epidermal growth factor; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal–related kinase; GFAT, glutamine:fructose-6-phosphate amidotransferase; MAPK, mitogen-activated protein kinase; PKC, protein kinase C; PMA, phorbol myristoyl acid; ROS, reactive oxygen species; Sp-1, stimulating protein 1; TGF, transforming growth factor; TRE, PMA responsive element; UDP-GlcNAc, uridine-5′-diphosphate-*N*-acetylglucosamine.

Diabetic nephropathy, one of the major causes of end-stage renal disease, is characterized by the thickening of glomerular and tubular basement membranes and the progressive expansion of the glomerular mesangium and the tubulointerstitium (1–3). The results of the Diabetes Control and Complications Trial have shown that strict prevention of hyperglycemia can prevent the onset and progression of diabetic nephropathy (4). In vivo and in vitro studies provide evidence that ambient high glucose induces the synthesis of extracellular matrix components (e.g., fibronectin) in glomerular and proximal tubular cells (5–9). There is ample evidence that the increased rate of glucose metabolism promotes the pathological changes in diabetic nephropathy (5,10). Because glucose transport is rate limiting for glucose metabolism, and because GLUT1, the main GLUT on mesangial cells, is a high-affinity, low-capacity transporter, mesangial glucose uptake appears to be essentially determined by the number of GLUT1 on the cell surface rather than ambient glucose concentration (11). Accordingly, experimental work has implicated the upregulation of GLUT1 as an important permissive factor for the increased matrix production by hyperglycemia (11–13). In cultured mesangial cells, ambient high glucose concentrations enhance GLUT1 expression and basal glucose uptake (12). Furthermore, the hyperglycemia-induced transforming growth factor (TGF)-β1 (13) and, similarly, angiotensin (ANG) II (14) stimulate GLUT1 expression. These data indicate an enhanced GLUT1 expression under diabetic conditions. Although little is known about the detailed cellular expression pattern of GLUT1 in the diabetic kidney (11), a recent study demonstrated a significant increase in GLUT1 protein in the renal cortex of diabetic animals (15). Furthermore, mesangial cells isolated from diabetic subjects have demonstrated enhanced GLUT1 expression and glucose uptake (16), and an *Xba*I polymorphism of the GLUT1 gene has been associated with development of the nephropathy in diabetic subjects (17).

To prove the role of GLUT1 overexpression on matrix synthesis, we established a mesangial cell model stably transfected with human GLUT1 (GT1 cells) (18). These cells showed a 5-fold increased glucose uptake, a 2.1-fold increase in lactate production, and enhanced expression and net deposition of fibronectin and other matrix proteins

when exposed to physiological levels of extracellular glucose (18). Moreover, antisense GLUT1 protects mesangial cells from high glucose–induced fibronectin production (19). Together, these data demonstrate that in mesangial cells, both increased glucose uptake with subsequent enhanced metabolism and hyperglycemic conditions activate fibronectin production.

The adverse effects of hyperglycemia in human and experimental diabetic nephropathy have been linked to the enhanced action of the prosclerotic cytokine TGF-β1 (20–22). The hyperglycemia-induced TGF-β stimulates the production of fibronectin and other matrix proteins in mesangial and other renal cells (8,9,22). Activation of protein kinase C (PKC) isoforms (7,10,23), the hexosamine biosynthetic pathway (24,25), and the extracellular signal–related kinase (ERK) 1 and 2 (26,27) and p38 mitogen-activated protein kinase (MAPK) pathway (28,29) have been implicated in the enhanced expression of TGF-β1 and matrix proteins. Of note, the hyperglycemia-enhanced generation of reactive oxygen species (ROS) has been linked to the activation of PKC (30) and the hexosamine pathway (31) and to enhanced TGF-β1 synthesis (31,32).

The increases in TGF-β1 and fibronectin production are mediated by an activation of the promoters of the corresponding genes. Promoters of the TGF-β1 (29,33) and the fibronectin gene (34) are stimulated by high glucose, and TGF-β1 itself is known to activate the fibronectin promoter (35). Transcription factors of the activating protein 1 (AP-1) family are activated by high glucose in mesangial cells (29,36,37) and mediate the transcriptional activation of the TGF-β1 and fibronectin genes (29,34,38).

In the present study, we investigated the molecular mechanism of the enhanced fibronectin synthesis induced by increased glucose uptake under normoglycemic conditions in GT1 cells. We focused our study on the possible role of TGF-β and AP-1 in the increased gene expression of the matrix protein fibronectin. Our data demonstrated a strong PKC-dependent activation of AP-1 that in turn induces TGF-β–independent fibronectin production.

RESEARCH DESIGN AND METHODS

Materials. Oligonucleotides were synthesized by Life Technologies (Karlsruhe, Germany). Cell culture media, supplements, Ultroser, and FCS were obtained from Gibco (Eggenstein, Germany); minocyclin, from Pan Systems (Aidenbach, Germany); Klenow enzyme and poly[d(I-C)], from Boehringer (Mannheim, Germany); bisindolylmaleimide I, phorbol myristoyl acid (PMA), anisomycin, and curcumin, from Sigma (Munich, Germany); calphostin, from Calbiochem (Schwalbach, Germany); PKC-peptide inhibitor, from Promega (Madison, WI); [α-^{32}P]dATP, from Hartmann (Braunschweig, Germany); antibodies against c-Jun (1694X), JunB (73X), JunD (74X), c-Fos (253X), stimulating protein 1 (Sp-1; 59X), and PKC-α (C-20) and -β1 (C-16), from Santa Cruz Technologies (Santa Cruz, CA); antibodies against phospho-p42/p44 MAPK thr-202/tyr-204, p42/p44 MAPK, phospho-activating transcription factor-2 thr-71, and phospho-p38 MAPK thr-180/tyr-182, from New England Biolabs (Schwalbach, Germany); the neutralizing TGF-β antibody (against TGF-β1, -β2, and -β5), from R&D Systems (Minneapolis, MN); the antibody against fibronectin, from Biomol (Hamburg, Germany); and the RL-2 antibody, from Dianova (Hamburg, Germany). The antibody against glutamine:fructose-6-phosphate amidotransferase (GFAT) has been previously described (39). The fluorescent probe CM-H_2DCFDA was obtained from Molecular Probes (Eugene, OR).

Cell culture. Rat mesangial cells stably transfected with the human GLUT1 transporter gene (GT1 cells) or bacterial β-galactosidase (as controls; LacZ cells) were cultured as previously described (18). For experimental purposes, RPMI 1640 contained 8 mmol/l glucose (corresponding to normal glucose levels in rats), 2% Ultroser, 2 mmol/l glutamine, 25 mmol/l HEPES, 100 units/ml penicillin, and 100 μg/ml streptomycin. For determination of TGF-β1 and fibronectin concentration in supernatants, medium without Ultroser or FCS was used.

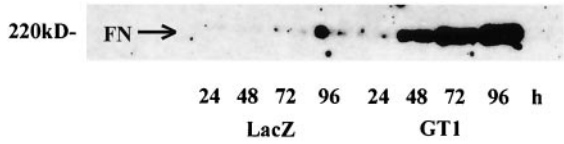

FIG. 1. Fibronectin secretion into the medium of LacZ and GT1 cells. In this study, 200 μl supernatant of LacZ and GT1 cells grown for 24, 48, 72, or 96 h were precipitated with acetone and separated on 7.5% SDS-PAGE. The antibody against fibronectin (FN) detected only the 220-kDa band, as indicated.

Determination of glucose, lactate, and DNA concentrations. In supernatants, glucose and lactate were measured with Ektachem Vitros systems (Ortho-clinical-diagnostics, Neckargmünd, Germany). DNA in total cell extracts was measured by fluorometry with bisbenzimidazol (24).

Northern blotting. RNA was prepared from LacZ and GT1 cells with the RNeasy Kit (Qiagen, Hilden, Germany). Total RNA (25 μg) was separated on a formaldehyde-containing agarose gel and transferred to a nylon membrane. Preparation and labeling of RNA probes for TGF-β1 and glyceraldehyde-3-phosphate dehydrogenase and hybridizations were performed as previously described (40).

Enzyme-linked immunosorbent assay for TGF-β1 and fibronectin. For quantification of the proteins, cell culture supernatants were collected after the indicated time points and stored at −80°C. Determination of total TGF-β1 protein was performed with the Quantikine Immunoassay (R&D Systems, Minneapolis, MN). Fibronectin was measured by the Quantimatrix human fibronectin enzyme-linked immunosorbent assay kit (ELISA) from Chemicon International (Temecula, CA).

Western blotting. For Western blotting studies, 6.0×10^6 cells were seeded onto 15-cm culture dishes with 15 ml experimental medium and harvested after 12, 24, or 48 h. Nuclear proteins were prepared as recently described (41). Preparation of total, cytosolic, and membrane fraction and detection of PKC isoforms was recently described (40). Western blotting was performed as previously described (29).

Electrophoretic mobility shift assay. The synthetic oligonucleotides phorbol myristic acid responsive element:5′-GATCTGTGACTCAGCGCGAG-3′ and Sp-1:5′-GATCTCGGGGAAAGGGGGCGGGCTCCCCAG-3′ were end-labeled with [α-^{32}P]dATP (3,000 Ci · mmol^{-1} · l^{-1}) and Klenow enzyme and were incubated with up to 5 μg nuclear protein in 20 μl of 7 mmol/l Hepes-KOH (pH 7.9), 100 mmol/l KCl, 3.6 mmol/l $MgCl_2$, and 10% glycerol on ice for 20 min. Next 0.05 mg/ml poly[d(I-C)] was added as an unspecific competitor. The samples were run on a 5% nondenaturating polyacrylamide gel in a buffer containing 25 mmol/l Tris-HCl (pH 8.0), 190 mmol/l glycine, and 1 mmol/l EDTA. Gels were dried and analyzed by autoradiography.

Determination of UDP-GlcNAc. Cell extracts were prepared and determination of uridine-5′-diphosphate-*N*-acetylglucosamine (UDP-GlcNAc) by capillary electrophoresis was performed as previously described (42).

Determination of reactive oxygen species. Cells were cultured with 8 or 30 mmol/l glucose in experimental medium for 48 h, loaded with 10 μmol/l CM-H_2DCFDA, incubated for 30 min at 37°C, and analyzed by fluorescence-activated cell sorting using FACScalibur (Becton-Dickinson, San Jose, CA).

Statistical analysis. Results presented are derived from at least three independent experiments. Means ± SE were calculated, and groups of data were compared using Student's t test. Statistical significance was set at $P < 0.05$.

RESULTS

Increased fibronectin production in GT1 cells was independent of TGF-β1. The increased flux through glycolysis in the GT1 cells was confirmed by a 3.2-fold increase in glucose consumption and a 3.6-fold higher lactate production after 72 h compared to LacZ cells, thereby indicating that glucose was almost completely (99%) metabolized to lactate. Furthermore, through Western blotting, we obtained a time-dependent accumulation of fibronectin protein in the supernatant of the GT1 cells, but observed only negligible fibronectin production in LacZ cells (Fig. 1). The results indicated that our cell culture conditions were suitable for investigating the

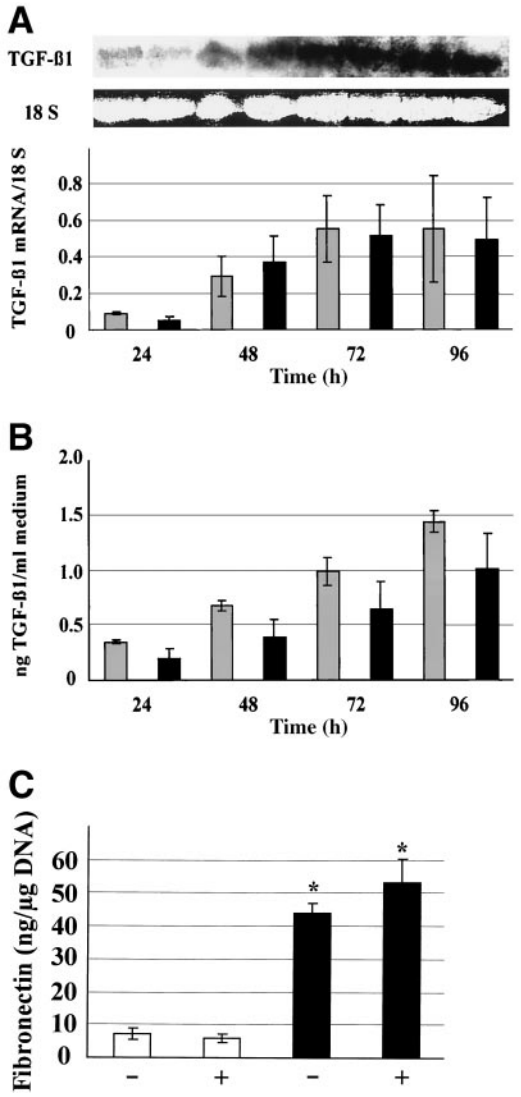

FIG. 2. Increased fibronectin production in GT1 cells is independent of TGF-β1. LacZ and GT1 cells were grown for 24, 48, 72, and 96 h. RNA was isolated as described in RESEARCH DESIGN AND METHODS and the supernatants were collected. *A*: Northern analysis of 25 µg total RNA of LacZ (▨) and GT1 (■) cells. After electrophoretic separation, the blots were probed for TGF-β1 using the digoxigenin-labeled RNA probe. Densities of the TGF-β1 mRNA signals were normalized to 18S RNA. One representative result out of four is shown. *B*: TGF-β1 protein was quantified in the supernatants of four independent experiments by ELISA. Bar graphs show the measured total TGF-β1 protein. Each value is expressed as the mean ± SE. ▨, LacZ cells; ■, GT1cells. *C*: LacZ (□) and GT1 cells (■) were grown for 48 h with (−) or without (+) 30 µg/ml anti-TGF-β1 antibody, after which the supernatants were collected. The amount of secreted fibronectin protein was determined by ELISA and related to the total DNA content of the cells. Bar graphs show the fibronectin/DNA ratio. Each value is expressed as the mean ± SE of three independent experiments, performed in duplicate. *P < 0.05 GT1 vs. LacZ.

mechanism of fibronectin induction by overexpression of GLUT1 in mesangial cells.

To study the hypothesis that the increased fibronectin production in GT1 cells is mediated by an enhanced expression of TGF-β1, the TGF-β1 mRNA levels in GT1 and LacZ cells were determined by Northern blotting. As shown in Fig. 2*A*, over a time course of 96 h, the amount of TGF-β1 mRNA was comparable in GT1 and LacZ cells, with similar increases after 48, 72, and 96 h. Because the secretion of the TGF-β1 protein is necessary for autocrine/paracrine bioactivity, the concentration of total TGF-β1 protein in the supernatant of the cells was measured by ELISA. We found no increased TGF-β1 protein production in GT1 cells (Fig. 2*B*), a finding that supports the observed TGF-β1 mRNA levels (Fig. 2*A*). In fact, the TGF-β1 protein production of GT1 cells appeared to be lower. The same result was obtained when TGF-β1 was related to cellular DNA (data not shown). These results indicated that overexpression of GLUT1, which leads to increased flux through glycolysis, does not induce TGF-β1 overexpression.

To test whether increased TGF-β1 bioactivity might account for the increased fibronectin synthesis, we studied the fibronectin production in the presence of neutralizing TGF-β antibodies. As shown in Fig. 2*C*, inhibition of TGF-β1 bioactivity did not prevent the increase in fibronectin protein in the supernatant of GT1 cells compared to LacZ cells, indicating that the increased fibronectin production was mediated by an TGF-β1−independent pathway.

Overexpression of GLUT1 increased nuclear levels of Jun proteins and the DNA-binding activity of AP-1 specifically. Because the rat fibronectin promoter contains a regulatory active AP-1 binding site (43), and the expression and activity of several AP-1 proteins were increased by high glucose, we studied the nuclear concentration of the AP-1 proteins c-Fos and c-Jun, JunB, and JunD in GT1 and LacZ cells by Western blotting. The amount of all three Jun proteins was clearly increased in GT1 cells compared to LacZ cells after 12, 24, and 48 h (Fig. 3*A*– *C*), except that no difference could be detected after 48 h in JunB proteins (Fig. 3*B*). The nuclear content of all three Jun proteins was lower in the GT1 cells cultured for 48 h compared to those cultured for 24 h. We found no increase of c-Fos in the nuclear extracts of GT1 cells after 12, 24, and 48 h compared to control cells (Fig. 3*D*). The 62-kDa band that was detected by the anti−c-Fos antibody was verified as c-Fos when after cells were stimulated with PMA, a strong activator of c-Fos expression, the 62 kDa band was clearly enhanced. Together, these data demonstrated that increases of glucose transport and metabolism by overexpression of GLUT1 in mesangial cells led to a strong elevation of the nuclear level of Jun proteins while the amount of c-Fos remained unchanged.

The increased amounts of Jun proteins in the nuclear extracts of GT1 cells resulted in enhanced DNA binding to a PMA responsive element (TRE) containing consensus AP-1 binding site. Through electrophoretic mobility shift assays (EMSAs), we found a strongly time-dependent increase in DNA-binding activity of nuclear proteins from GT1 cells compared to LacZ cells (Fig. 3*E*). Although the difference between GT1 and LacZ cells was excessive at 12 and 24 h, the difference was less pronounced after 48 h, a finding that supports the lower nuclear amount of Jun proteins at this time point (Fig. 3*A*– *C*). Using specific antibodies against AP-1 proteins, we characterized the DNA-binding protein complex in GT1 cells as a heterodimer consisting of c-Fos and the Jun proteins c-Jun, Jun B, and Jun D (Fig. 3*F*).

The activation of AP-1 in the GT1 cells was specific, as we found no enhanced nuclear content or DNA-binding

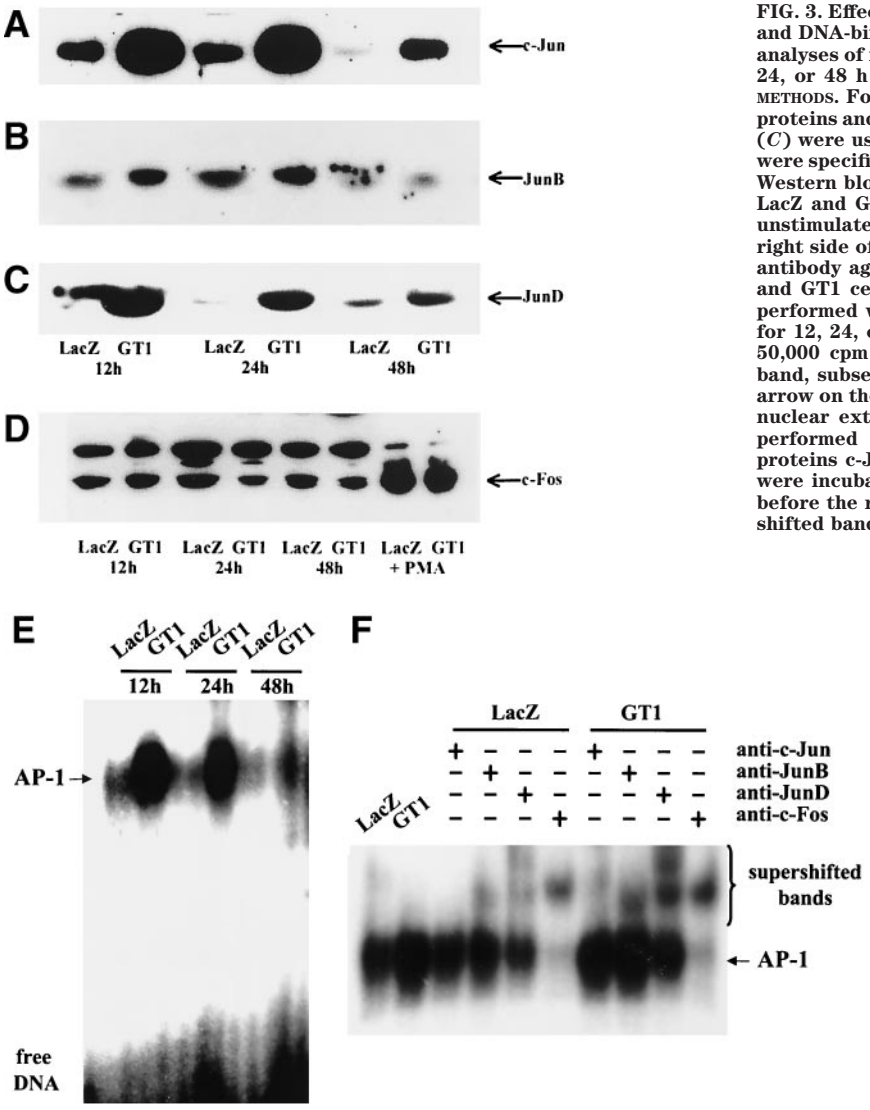

FIG. 3. Effect of overexpression of GLUT1 on the nuclear amounts and DNA-binding activity of AP-1 proteins. *A* **and** *C*: **Western blot analyses of nuclear extracts from LacZ and GT1 cells grown for 12, 24, or 48 h were performed as described in** RESEARCH DESIGN AND METHODS. **For immunodetection of AP-1 proteins, 20 μg of nuclear proteins and antibodies recognizing c-Jun (*A*), JunB (*B*), and JunD (*C*) were used. The antibodies against c-Jun, and JunB, and JunD were specific and not cross-reactive with the other Jun proteins.** *D*: **Western blot analyses for c-Fos with 20 μg of nuclear proteins of LacZ and GT1 cells stimulated with 0.5 μmol/l PMA for 6 h and unstimulated cells grown for the indicated times. The arrow on the right side of the figure marks the 62-kDa protein identified by the antibody against c-Fos.** *E*: **Binding of nuclear proteins from LacZ and GT1 cells to the AP-1 site. Mobility shift experiments were performed with nuclear extracts from LacZ and GT1 cells grown for 12, 24, or 48 h. Nuclear proteins (5 μg) were incubated with 50,000 cpm of the ^{32}P-labeled oligonucleotide TRE. The shifted band, subsequently identified as AP-1 complex, is marked by the arrow on the left site of the figure.** *F*: **Supershift experiments with nuclear extracts from LacZ and GT1 cells grown for 48 h were performed with specific antibodies against cFos and the Jun proteins c-Jun, Jun B, and Jun D. Then 2 μg of specific antibody were incubated with the binding reaction mixture for 1 h on ice before the radiolabeled DNA sequence was added. Specific supershifted bands are indicated by brackets.**

activity of other transcription factors that have been linked to high glucose–induced promoter activation, such as Sp-1 (25,31) or cAMP-responsive element binding protein (CREB)/activating transcription factor (ATF) 1 (34). Determination of Sp-1 in the same nuclear extracts that were used for studies on AP-1 revealed changes in neither the amount of Sp-1 protein (Fig. 4*A*) nor in DNA-binding activity to the Sp-1 site (Fig. 4*B*). Specificity of Sp-1 DNA binding was determined by mutated Sp-1 site and supershift analysis (data not shown). Similarly, we found no changes in the nuclear amounts of the AP-1–related proteins ATF-1, ATF-2, or CREB by Western blotting or the binding of nuclear proteins to a cAMP-responsive element (CRE), which contains the consensus binding sequence for ATF and CREB proteins (data not shown). These data indicated the specificity of the AP-1 activation in GT1 cells.

The increase in fibronectin expression was inhibited in a dosage-dependent manner by the AP-1 inhibitor curcumin. Our studies revealed that TGF-β1 is not responsible for the enhanced expression of fibronectin in the GT1 cells. Furthermore, we observed a strong activation of AP-1. Therefore, we tested whether the enhanced expression and DNA-binding activity of AP-1 would mediate the accumulation of fibronectin in the supernatant of the GT1 cells by the addition of curcumin. Curcumin is known to specifically inhibit the activation and DNA binding of AP-1 (44,45). The strong increase of fibronectin produced by the GT1 cells compared to LacZ cells after 48 h (41.9 ± 7.1 vs. 5.4 ± 1.1 ng/μg DNA) was reduced by curcumin in a dosage-dependent manner, whereas LacZ cells were not affected (Fig. 5). At a concentration of 20 μmol/l curcumin, the fibronectin production was decreased by 66% compared to GT1 cells cultured without the AP-1 inhibitor. These data indicated that activation of AP-1 is necessary for the enhanced expression of fibronectin observed in the GT1 cells.

Inhibition of PKC prevented the induction of AP-1 and enhanced fibronectin production. Because it has been reported that the hyperglycemia-induced activation of PKC isoforms is involved in the increased production of fibronectin, we studied the effect of two inhibitors of PKC, calphostin and a peptide inhibitor specific for PKC isoforms α and β, on the induction of AP-1 and the fibronectin production in the GT1 cells. The addition of 1,000 nmol/l calphostin or 10 μmol/l peptide inhibitor to the GT1 cells for 24 h reduced the nuclear amounts of c-Jun and JunD (Fig. 6*A*) and consequently the DNA-binding activity of the nuclear extracts to the AP-1 consensus sequence (Fig. 6*B*).

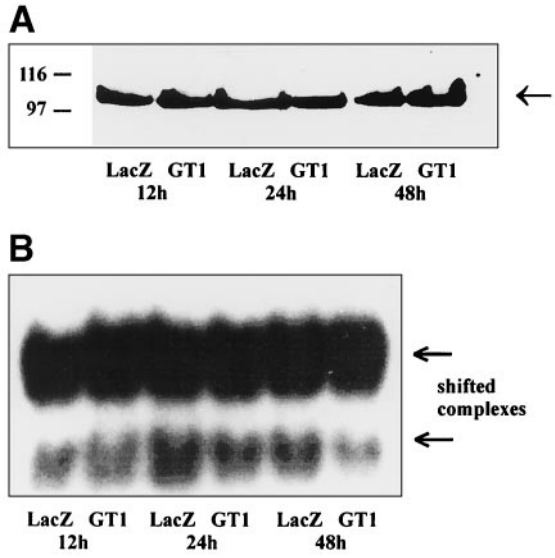

FIG. 4. Effect of overexpression of GLUT1 on nuclear Sp-1 levels and Sp-1 activation. *A*: Western analysis of nuclear extracts of LacZ and GT1 cells grown for 12, 24, and 48 h. Then 40 μg of nuclear proteins were separated on a 7.5% SDS-PAGE, and Sp-1 was identified with a specific antibody. Molecular weight markers are indicated on the left side of the figure; Sp-1 protein is marked by the arrow on the right side. *B*: Mobility shift assay performed with 3 μg of nuclear proteins of LacZ and GT1 cells using 50,000 cpm of the ^{32}P-labeled Sp-1 consensus oligonucleotide. The arrows on the right side of the figure mark the two shifted bands. The lower, less intense band did not react with an antibody against Sp-1, whereas the upper band contains Sp-1 (not shown). The free, unbound DNA is not shown.

Furthermore, the fivefold increased production of fibronectin in GT1 cells was reduced to the level of LacZ cells (Fig. 6*C*). Treatment of the cells with 100 nmol/l calphostin showed a minor reduction of c-Jun and JunD protein content, DNA-binding activity of an AP-1 complex, and fibronectin production of the GT1 cells. In LacZ cells, the addition of calphostin or the peptide inhibitor also decreased the amount of Jun proteins and the DNA-binding activity of AP-1, but had no effect on fibronectin production (Fig. 6*A*–C). The effect on the nuclear amount of JunB was not tested, as the JunB protein content in nuclear extracts was very low and the increase in GT1 cells was much less pronounced when compared to c-Jun or JunD.

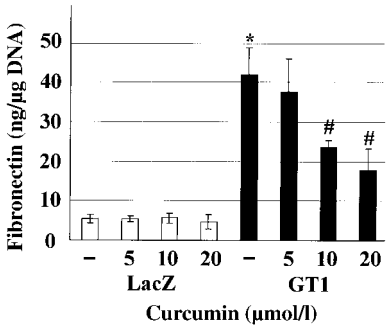

FIG. 5. Effect of curcumin on fibronectin synthesis in GT1 cells. LacZ and GT1 cells were grown for 48 h with or without the indicated concentrations of curcumin, after which supernatants were collected. The amount of secreted fibronectin protein was determined by ELISA and related to the total DNA content of the cells. Bar graphs show the fibronectin/DNA ratio. Each value represents the mean ± SE of three independent experiments, performed in duplicate. *P < 0.05 GT1 vs. LacZ without curcumin; #P < 0.05 GT1 with curcumin vs. GT1 without curcumin.

Because the peptide inhibitor is specific for PKC isoforms α and β, we studied the amount and translocation of PKC-α and -β1 (PKC-β2 was not expressed in GT1 and LacZ cells; data not shown) by Western blotting. PKC-α and -β1 were increased in the total, cytosolic, and membrane extracts of the GT1 cells compared to LacZ cells (Fig. 6*D*). The enhanced translocation of PKC-α and -β1 indicated a participation of these PKC isoforms in AP-1 activation and subsequent enhanced fibronectin production in the GT1 cells.

No activation of other TGF-β1–inducing signaling pathways in the GT1 cells observed. In addition to the PKC-dependent pathways, the hexosamine pathway and the ERK-1 and -2, and p38 MAPK pathways were activated by ambient high glucose and were linked to the development of late diabetic complications, particularly to the high glucose–enhanced expression of TGF-β1 in mesangial cells. Therefore, we asked whether a missing activation of one or more of these pathways in the GT1 cells might explain the missing increase in TGF-β1 synthesis.

We studied the flux through the hexosamine pathway by measuring its end product UDP-GlcNAc, and by determining the endogenous levels of O-GlcNAc–modified nuclear proteins and the cellular levels of the key enzyme GFAT by Western blotting (Fig. 7). Assessment of intracellular UDP-GlcNAc by capillary electrophoresis revealed that the concentration of the hexosamine pathway end product was unchanged or even reduced in the GT1 cells compared to LacZ cells after 24, 48, 72, and 96 h when related to the DNA content (Fig. 7*A*) or cell number (data not shown). This observation was supported by the reduced amount of *O*-GlcNAc–modified proteins in the nuclear extracts of the GT1 cells after 24 and 48 h (Fig. 7*B*). Furthermore, we found reduced endogenous levels of GFAT protein in the cytosolic extracts of GT1 cells harvested after 24 and 48 h compared to LacZ cells (Fig. 7*C*), which may have contributed to the low activity of the hexosamine pathway in GT1 cells.

We then studied the possible activation of the p38 MAPK and ERK-1 and -2 pathways in GT1 cells by using specific phosphopeptide antibodies. The intensities of the bands detected by the phosphospecific antibodies against p38 MAPK and ATF-2 were not different in LacZ and GT1 cells (Fig. 8*A*). Stimulation of the cells with anisomycin, an activator of the p38 MAPK pathway, resulted in a strongly increased intensity of the respective bands. Furthermore, we studied the phosphorylation of the MAPK ERK-1 and -2, which contribute to the activation of this MAPK pathway. No enhanced levels of phosphorylated ERK-1 or -2 could be detected in GT1 cells compared to LacZ cells, whereas stimulation of the cells with epidermal growth factor (EGF) resulted in increased intensity of the bands, detected by the phosphospecific antibodies against ERK-1 and -2 (Fig. 8*B*).

ROS as signaling molecules have been implicated in the activation of the hexosamine and p38 MAPK pathways. Therefore we studied the production of ROS in the GT1 and LacZ cells by fluorescence activated cell sorting. After a 48-h cell culture with ambient 8 mmol/l glucose, no increased fluorescence intensity signal could be detected

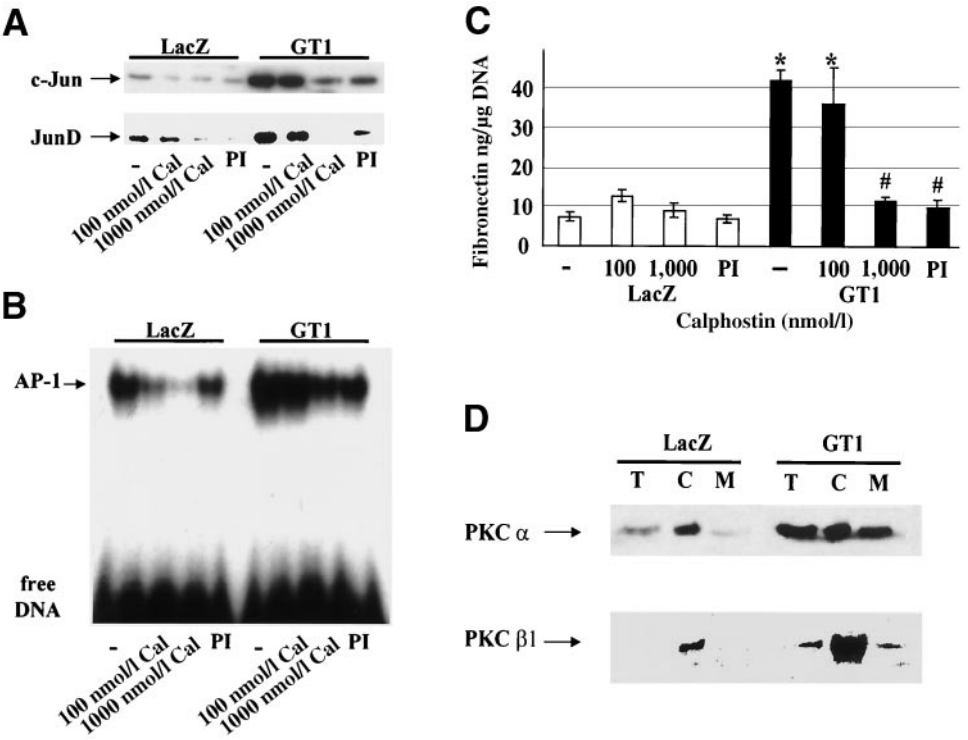

FIG. 6. Inhibition of PKC isoforms prevents the induction of AP-1 and the enhanced fibronectin production. LacZ and GT1 cells were grown in the presence of 100 or 1,000 nmol/l calphostin (Cal) or 10 µmol/l PKC peptide inhibitor (PI). Nuclear extracts were prepared after 24 h of cell culture and total extracts and cytosolic or membrane fraction were prepared after 48 h of cell culture. Supernatants were collected after 48 h. *A*: Western analysis of 15 µg of nuclear proteins using the anti-c-Jun and anti-JunD antibodies. Detected Jun proteins are marked by arrows. *B*: DNA-binding activity of the nuclear proteins to the radiolabeled TRE containing AP-1 consensus sequence as detected by EMSA. AP-1 specific band is marked by arrow. *C*: The amount of secreted fibronectin protein was determined by ELISA and related to the total DNA content of the cells. Bar graphs show the fibronectin/DNA ratio. Each value represents the mean ± SE of three independent experiments, performed in duplicate. *$P < 0.05$ GT1 vs. LacZ without inhibitors; #$P < 0.05$ GT1 with versus inhibitors. *D*: Western analysis of the total extract (T) and the cytosolic (C) and membrane fraction (M) using antibodies against PKC isoform α and β1(arrows).

in the GT1 cells compared to LacZ cells, indicating no increased generation of ROS by overexpressing GLUT1 (Fig. 8*C*). As expected, exposure of LacZ cells to 30 mmol/l glucose for 48 h resulted in a 1.6-fold increase in fluorescence signal compared to exposure to 8 mmol/l glucose, indicating that oxidative stress is caused by ambient high glucose. In the GT1 cells, ambient 30 mmol/l glucose failed to generate a fluorescence signal above that of the GT1 cells cultured in 8 mmol/l glucose.

DISCUSSION

This is the first report demonstrating that in mesangial cells, excessive transport of glucose by overexpressing GLUT1 in the absence of ambient high glucose activates synthesis of fibronectin independent of TGF-β1. Three distinct lines of evidence support this conclusion: *1*) TGF-β1 mRNA levels were unchanged in mesangial cells overexpressing GLUT1; *2*) the concentration of the secreted TGF-β1 protein in the supernatant of the GT1 cells was not increased compared to control cells; and *3*) the addition of neutralizing antibodies against bioactive TGF-β1 did not reduce the strongly increased concentration of fibronectin in the supernatant of the GT1 cells. Moreover, our results indicated that fibronectin production in the GT1 cells was mediated through activation of PKC and AP-1. Overexpression of GLUT1 increases the expression and content in the membrane fraction of PKC isoforms α (46) and β1, indicating an activation of these isoforms. Inhibition of PKC with two different inhibitors, one of them specific for isoforms α and β1, blocked the excessive activation of AP1 in the GT1 cells. This activation was demonstrated by the strong increase of c-Jun, JunD, and JunB proteins, subsequently leading to increased DNA-binding activity of the AP-1 complex and to enhanced fibronectin production. The finding that fibronectin accumulation is prevented by inhibition of PKC provides very clear evidence for the following sequential signaling pathway: excessive glucose uptake and metabolism → activation of PKC → activation of AP-1 → fibronectin accumulation.

The second main observation from this study is the missing upregulation of TGF-β1, despite the increases in glucose uptake and metabolism and the activation of PKC and AP-1. We could clearly demonstrate that the participation of other high glucose−inducible pathways is necessary for the induction of TGF-β1 expression by ambient high glucose as well as by increased glucose uptake. Specifically, the activation of the hexosamine (24,25) and the p38 (28) and ERK-1 and -2 MAPK pathways (26,27) by high glucose, which has been demonstrated by several groups in vivo and in vitro and has been linked to the induction of TGF-β1 expression (24,27,29), is completely missing in the GT1 cells. In addition, after comparing the activation of AP-1 proteins in the GT1 and mesangial cells exposed to ambient high glucose, we found no increased nuclear levels of c-Fos, whereas the expression of c-Fos is induced by high extracellular glucose conditions (29,37) and is implicated in enhanced TGF-β1 gene activation (29). Therefore, our results strongly suggest that overexpression of GLUT1 in mesangial cells exposed to physiologically occurring glucose concentrations and exposure of mesangial cells to high extracellular glucose do not induce the same biochemical pathways and that activation of classical PKC and Jun proteins alone is not sufficient to induce TGF-β1 synthesis.

This observation is supported by the lack of increased ROS production in GT1 cells under normal and high glucose conditions, compared to the generation of ROS in control cells under high glucose conditions. Apparently, the chronically excessive glucose uptake in GT1 cells did not enhance the flux of glucose metabolites into the electron transport chain, as almost all glucose was metab-

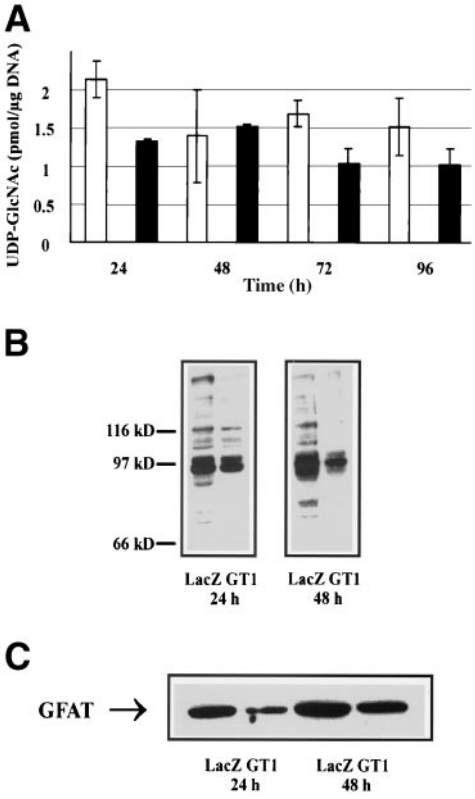

FIG. 7. The hexosamine pathway is not activated in GT1 cells. LacZ and GT1 cells were grown for the indicated time points and extracts were prepared as described in RESEARCH DESIGN AND METHODS. *A*: The endogenous concentration of UDP-GlcNAc was determined by capillary electrophoresis and related to the total DNA content of the cells. Bar graphs show the UDP-GlcNAc/DNA ratio. Each value represents the mean ± SE of three independent experiments performed as triplicate. □, LacZ; ■, GT1. *B*: Western analyses of 15 µg of nuclear proteins of LacZ and GT1 cells using an antibody against *O*-GlcNAc-modified proteins. Molecular weight markers are indicated on the left side of the figure. *C*: The endogenous level of GFAT was determined by Western analysis with 20 µg of cytosolic extract. Cytosolic extracts were harvested from 2.0×10^5 cells seeded onto one 6-well dish after 24 and 48 h of cell culture with 150 µl lysis buffer (Roche, Mannheim, Germany). The band detected by the antibody representing GFAT protein is indicated by an arrow on the left side of the figure.

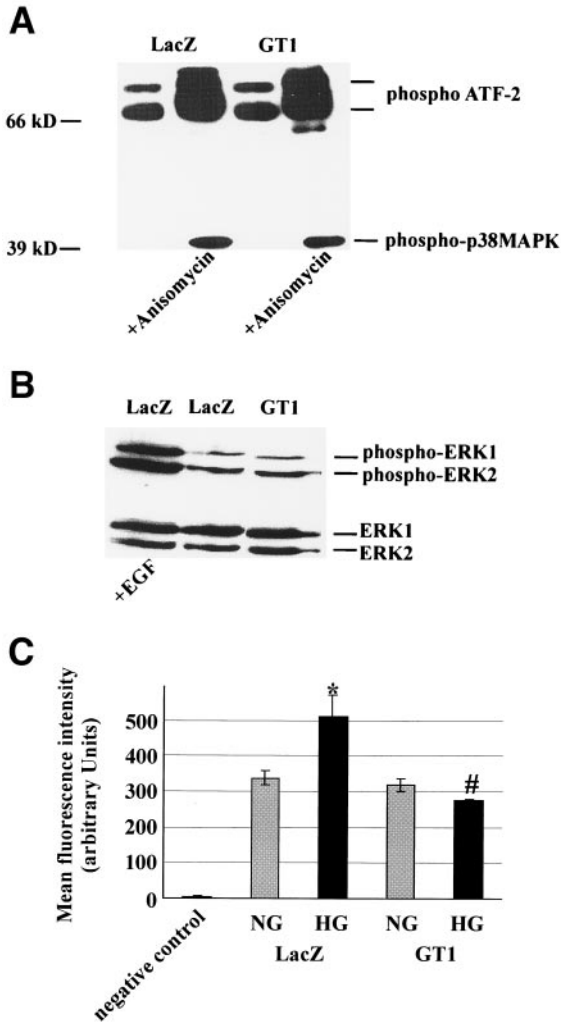

FIG. 8. Effect of overexpression of GLUT1 on p38 MAPK and ERK-1 and -2 pathways and on intracellular oxidant stress. *A*: In this study, 15 µg protein of nuclear extracts obtained from LacZ and GT1 cells grown for 24 h with or without stimulation with 10 µg/ml anisomycin for 1 h as indicated were analyzed by Western blotting using phosphospecific antibodies against the phosphorylated forms of p38 MAPK and ATF-2. Molecular weight markers are indicated on the left side of the figure and the phosphorylated forms of p38 MAPK and ATF-2 are marked on the right side of the figure. *B*: For EGF studies, 40 µg protein of cellular extracts obtained from LacZ and GT1 cells with or without stimulation with EGF (10 ng/ml) for 5 min as indicated were analyzed by Western blotting using phosphospecific antibodies against the phosphorylated forms of ERK-1 and -2 MAPK (*upper panel*). Total amount of ERK-1 and -2 are shown in the lower panel. *C*: Fluorescence signals generated by treating LacZ and GT1 cells with redox-sensitive dye CM-H_2DCFDA. Before addition of the dye, cells were cultured in experimental medium containing 8 mmol/l (NG) or 30 mmol/l glucose (HG) for 48 h. Graphs summarize the fluorescence intensity of CM-H_2DCFDA–loaded LacZ and GT1 cells. Each value represents the mean ± SE of three independent experiments. *$P < 0.05$ LacZ (HG) vs. LacZ (NG); #$P < 0.05$ GT1 (HG) vs. LacZ (HG).

olized to lactate. This prevented increased ROS production in the mitochondria, a result that was recently linked to the development of diabetic late complications in a study of endothelial cells (30,31). The absence of oxidative stress can explain the missing upregulation of the redox-sensitive hexosamine and the p38 MAPK pathways in the GT1 cells. Moreover, this finding uncovers a glucose-dependent, oxidative stress–independent mechanism of fibronectin production in mesangial cells.

Our findings in GLUT1-overexpressing mesangial cells are relevant for the in vivo situation in two ways: first, in contrast to other cells (e.g., endothelial cells) (47), mesangial cells respond to high glucose concentrations with increased GLUT1 levels (12), a finding also seen in mesangial cells obtained from diabetic patients (16). Second, they reflect the activated signaling pathways in situations where blood glucose concentration are normal or near normal, but glucose uptake is still increased because of diabetes-induced cytokine levels (e.g., ANG, insulin-like growth factor 1, pseudohypoxia).

Various in vitro and in vivo studies have shown that inhibition of TGF-β1 by, for example, treatment with monoclonal anti–TGF-β1 antibody (22) prevents the increase in glomerular matrix proteins, indicating that TGF-β1 is a major causative factor in diabetic nephropathy. Therefore, it may well be that blockade of TGF-β leads to a reduction of other, TGF-β1–induced events occurring in diabetic nephropathy, such as cellular hyperplasia or expression of other matrix proteins, and possibly leads to the inhibition of hyperglycemia- (12,13) or ANG-II–induced (14) GLUT1 overproduction, thus interrupting the previously proposed TGF-β1–GLUT1 axis (11). Our

finding that the excessively increased flux through glycolysis stimulates the production of extracellular matrix proteins via AP-1 activation may be relevant for other pathological situations with massive overexpression of GLUT1, such as in tumor cells (48) or hypoxic tissues (49).

In summary, the mesangial cells overexpressing GLUT1 may serve as a good model for distinguishing between the effects of ambient high glucose and increased glucose uptake and subsequent enhanced metabolism in physiological glucose concentrations. This might be important because both acute hyperglycemia and normal or mild glycemia with increased GLUT1 caused by, for example, TGF-β1, ANG-II, hypoxia, or pseudohypoxia, may be present in diabetic patients (50). Therefore, both, TGF-β1−dependent as well as TGF-β1−independent pathways may contribute to mesangial matrix accumulation, one major hallmark in the development of diabetic nephropathy. Furthermore, from this and other studies it appears that the efficiency of inhibitors of tissue fibrosis needs to be evaluated on both pathways.

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REFERENCES

1. Osterby R: Glomerular structural changes in type 1 (insulin-dependent) diabetes mellitus: causes, consequences, and prevention. *Diabetologia* 35:803–812, 1992
2. Mauer SM, Steffes MW, Ellis EN, Sutherland DF, Brown DM, Goetz FC: Structural-functional relationships in diabetic nephropathy. *J Clin Invest* 74:1143–1155, 1984
3. Nerlich A, Schleicher E: Immunohistochemical localization of extracellular matrix components in human diabetic glomerular lesions. *Am J Pathol* 139:889–899, 1991
4. The Diabetes Control and Complications: Trial research group: The effects of intensive insulin treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N Engl J Med* 329:977–986, 1993
5. Ayo SH, Radnik RA, Garoni JA, Glass WF, Kreisberg JI: High glucose causes an increase in extracellular matrix proteins in cultured mesangial cells. *Am J Pathol* 136:1339–1348, 1990
6. Fukui M, Nakamura T, Ebihara I, Shirato I, Tomino Y, Koide H: ECM gene expression and its modulation by insulin in diabetic rats. *Diabetes* 41: 1520–1527, 1992
7. Studer RK, Craven PA, DeRubertis FR: Role for protein kinase C in the mediation of increased fibronectin accumulation by mesangial cells grown in high-glucose medium. *Diabetes* 42:118–126, 1993
8. Ziyadeh FN, Sharma K, Ericksen M, Wolf G: Stimulation of collagen gene expression and protein synthesis in murine mesangial cells by high glucose is mediated by autocrine activation of transforming growth factor-beta. *J Clin Invest* 93:536–542, 1994
9. Kolm V, Sauer U, Olgemöller B, Schleicher E: High glucose-induced TGF-β1 regulates mesangial production of heparan sulfate proteoglycan. *Am J Physiol* 270:F812–F821, 1996
10. Ayo SH, Radnik R, Garoni JA, Troyer DA, Kreisberg JI: High glucose increases diacylglycerol mass and activates protein kinase C in mesangial cell cultures. *Am J Physiol* 261:F571–F577, 1991
11. Mogyorosi A, Ziyadeh FN: GLUT1 and TGF-β: the link between hyperglycemia and diabetic nephropathy. *Nephrol Dial Transplant* 14:2827–2829, 1999
12. Heilig CW, Liu Y, England RL, Freytag SO, Gilbert JD, Heilig KO, Zhu M, Concepcion LA, Brosius III FC: D-Glucose stimulates mesangial cell GLUT1 expression and basal and IGF-1-sensitive glucose uptake in rat mesangial cells. *Diabetes* 46:1030–1039, 1997
13. Inoki K, Haneda M, Maeda S, Koya D, Kikkawa R: TGF-β1 stimulates glucose uptake by enhancing GLUT1 expression in mesangial cells. *Kidney Int* 55:1704–1712, 1999
14. Quinn LA, McCumbee WD: Regulation of glucose transport by angiotensin II and glucose in cultured vascular smooth muscle cells. *J Cell Physiol* 177:94–102, 1998
15. D'Agord Schaan B, Lacchini S, Bertoluci MC, Irigoyen MC, Machado UF, Schmid H: Increased renal GLUT1 abundance and urinary TGF-beta 1 in streptozotocin-induced diabetic rats: implications for the development of nephropathy complicating diabetes. *Horm Metab Res* 33:664–669, 2001
16. Liu ZH, Chen ZH, Li Y-J, Liu D, Li LS: Phenotypic alterations of live mesangial cells in patients with diabetic nephropathy obtained from renal biopsy specimen (Abstract). *J Am Soc Nephrol* 10:A0669, 1999
17. Hodgkinson AD, Millward BA, Demaine AG: Polymorphisms of the glucose transporter (GLUT1) gene are associated with diabetic nephropathy. *Kidney Int* 59:985–989, 2001
18. Heilig CW, Conception LA, Riser BL, Freytag SO, Zhu M, Cordes P: Overexpression of glucose transporters in rat mesangial cells cultured in a normal glucose milieu mimics the diabetic phenotype. *J Clin Invest* 96:1802–1814, 1995
19. Heilig CW, Kreisberg JI, Freytag S, Murakami T, Ebina Y, Guo L, Heilig K, Loberg R, Qu X, Jin Y, Henry D, Brosius FC III: Antisense GLUT-1 protects mesangial cells from glucose induction of GLUT-1 and fibronectin expression. *Am J Physiol Renal Physiol* 280:F657–F666, 2001
20. Yamamoto T, Nakamura T, Noble NA, Ruoslahti E, Border WA: Expression of transforming growth factor β is elevated in human and experimental diabetic nephropathy. *Proc Natl Acad Sci U S A* 90:1814–1818, 1993
21. Iwano M, Kubo A, Nishino T, Sato H, Nishioka H, Akai Y, Kurioka H, Fujii Y, Kanauchi M, Shiiki S, Dohi K: Quantification of glomerular TGF-β1 mRNA in patients with diabetes mellitus. *Kidney Int* 49:1120–1126, 1996
22. Ziyadeh FN, Hoffman BB, Iglesias-De la Cruz M, Han D, Hong W, Isono M, Chen S, McGowan T, Sharma K: Long-term prevention of renal insufficiency, excess matrix gene expression, and glomerular mesangial matrix expansion by treatment with monoclonal antitransforming growth factor-β antibody in db/db diabetic mice. *Proc Natl Acad Sci U S A* 97:8015–8020, 2000
23. Koya D, Haneda M, Nakagawa H, Isshiki K, Sato H, Maeda S, Sugimoto T, Yasuda H, Kashiwagi A, Ways DK, King GL, Kikkawa R: Amelioration of accelerated diabetic mesangial expansion by treatment with a PKC beta inhibitor in diabetic db/db mice, a rodent model for type 2 diabetes. *FASEB J* 14:439–447, 2000
24. Kolm-Litty V, Sauer U, Nerlich A, Lehmann R, Schleicher ED: High glucose-induced transforming growth factor β1 production is mediated by the hexosamine pathway in porcine glomerular mesangial cells. *J Clin Invest* 101:160–169, 1998
25. Goldberg HJ, Scholey J, Fantus IG: Glucosamine activates the plasminogen activator inhibitor 1 gene promoter through Sp1 DNA binding sites in glomerular mesangial cells. *Diabetes* 49:863–871, 2000
26. Haneda M, Araki S, Togawa M, Sugimoto T, Isono M, Kikkawa R: Mitogen-activated protein kinase cascade is activated in glomeruli of diabetic rats and glomerular mesangial cells cultured under high glucose conditions. *Diabetes* 46:847–853, 1997
27. Isono M, Cruz MC, Chen S, Hong SW, Ziyadeh FN: Extracellular signal-regulated kinase mediates stimulation of TGF-β1 and matrix by high glucose in mesangial cells. *J Am Soc Nephrol* 11:2222–2230, 2000
28. Igarashi M, Wakasaki H, Takahara N, Ishii H, Yang YJ, Yamauchi T, Kuboki K, Meier M, Rhodes CJ, King GL: Glucose or diabetes activate p38 mitogen-activated protein kinase via different pathways. *J Clin Invest* 103:185–195, 1999
29. Weigert C, Sauer U, Brodbeck K, Pfeiffer A, Haering HU, Schleicher ED: AP-1 proteins mediate hyperglycemia-induced activation of the human TGF-β1 promoter in mesangial cells. *J Am Soc Nephrol* 11:2007–2016, 2000
30. Nishikara T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, Yorek MA, Beebe D, Oates PJ, Hammes HP, Giardino I, Brownlee M: Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature* 404:787–790, 2000
31. Du XL, Edelstein D, Rossetti L, Fantus IG, Goldberg H, Ziyadeh F, Wu J, Brownlee M: Hyperglycemia-induced mitochondrial superoxide overproduction activates the hexosamine pathway and induces plasminogen activator inhibitor-1 expression by increasing Sp1 glycosylation. *Proc Natl Acad Sci U S A* 97:12222–12226, 2000
32. Iglesias-De La Cruz MC, Ruiz-Torres P, Alcami J, Diez-Marques L, Ortega-Velazquez R, Chen S, Rodriguez-Puyol M, Ziyadeh FN, Rodriguez-Puyol D: Hydrogen peroxide increases extracellular matrix mRNA through TGF-beta in human mesangial cells. *Kidney Int* 59:87–95, 2001
33. Hoffman BB, Sharma K, Zhu Y, Ziyadeh FN: Transcriptional activation of

transforming growth factor-β1 in mesangial cell culture by high glucose concentration. *Kidney Int* 54:1107–1116, 1998
34. Kreisberg JI, Garoni JA, Radnik R, Ayo SH: High glucose and TGF-β1 stimulate fibronectin gene expression through a cAMP response element. *Kidney Int* 46:1019–1024, 1994
35. Hocevar BA, Brown TL, Howe PH: TGF-β induces fibronectin synthesis through a c-Jun N-terminal kinase-dependent, Smad4-independent pathway. *EMBO J* 18:1345–1356, 1999
36. Wilmer WA, Cosio FG: DNA binding of activator protein-1 is increased in human mesangial cells cultured in high glucose concentrations. *Kidney Int* 53:1172–1181, 1998
37. Kreisberg JI, Radnik RA, Ayo SH, Garoni J, Saikumar P: High glucose elevates c-fos and c-jun transcripts and proteins in mesangial cell cultures. *Kidney Int* 46:105–112, 1994
38. Kim S-J, Angel P, Lafyatis R, Hattori K, Kim KY, Sporn MB, Karin M, Roberts AB: Autoinduction of transforming growth factor β1 is mediated by the AP-1 complex. *Mol Cell Biol* 10:1492–1497, 1990
39. Nerlich AG, Sauer U, Kolm-Litty V, Wagner E, Koch M, Schleicher ED: Expression of glutamine:fructose-6-phosphate amidotransferase in human tissues: evidence for high variability and distinct regulation in diabetes. *Diabetes* 47:170–178, 1998
40. Ceol M, Gambaro G, Sauer U, Baggio B, Anglani F, Forino M, Facchin S, Bordin L, Weigert C, Nerlich A, Schleicher ED: Glycosaminoglycan therapy prevents TGF-β1 overexpression and pathologic changes in renal tissue of long-term diabetic rats. *J Am Soc Nephrol* 11:2324–2336, 2000
41. Netzker R, Weigert C, Brand K: Role of the stimulatory proteins Sp1 and Sp3 in the regulation of transcription of the rat pyruvate kinase M gene. *Eur J Biochem* 245:174–181, 1997
42. Lehmann R, Huber M, Beck A, Schindera T, Rinkler T, Houdali B, Weigert C, Haring HU, Voelter W, Schleicher ED: Simultaneous, quantitative analysis of UDP-N-acetylglucosamine, UDP-N-acetylgalactosamine, UDP-glucose and UDP-galactose in human peripheral blood cells, muscle biopsies and cultured mesangial cells by capillary zone electrophoresis. *Electrophoresis* 21:3010–3015, 2000
43. Tamura K, Nyui N, Tamura N, Fujita T, Kihara M, Toya Y, Takasaki I, Takagi N, Ishii M, Oda K, Masatsugu H, Umemura S: Mechanism of angiotensin II-mediated regulation of fibronectin gene in rat vascular smooth muscle cells. *J Biol Chem* 41:26487–26496, 1998
44. Ahmad M, Theofanidis P, Medford RM: Role of the activating protein-1 in the regulation of the vascular cell adhesion molecule-1 gene expression by tumor necrosis factor-α. *J Biol Chem* 273:4616–4621, 1998
45. Greenwel P, Inagaki Y, Hu W, Walsh M, Ramirez F: Sp1 is required for the early response of alpha2(I) collagen to transforming growth factor-beta1. *J Biol Chem* 272:19738–19745, 1997
46. Henry DN, Busik JV, Brosius III FC, Heilig CW: Glucose transporters control gene expression of aldose reductase, PKCα, and GLUT1 in mesangial cells in vitro. *Am J Physiol* 277:F97–F104, 1999
47. Alpert E, Gruzman A, Totary H, Kaiser N, Reich R, Sasson S: A natural protective mechanism against hyperglycaemia in vascular endothelial and smooth-muscle cells: role of glucose and 12-hydroxyeicosatetraenoic acid. *Biochem J* 362:413–422, 2002
48. Burstein DE, Reder I, Weiser K, Tong T, Pritsker A, Haber RS: GLUT1 glucose transporter: a highly sensitive marker of malignancy in body cavity effusions. *Mol Pathol* 11:392–396, 1998
49. Zhang JZ, Behrooz A, Ismail-Beigi F: Regulation of glucose transport by hypoxia. *Am J Kidney Dis* 34:189–202, 1999
50. Meyer C, Stumvoll M, Nadkarni V, Dostou J, Mitrakou A, Gerich J: Abnormal renal and hepatic glucose metabolism in type 2 diabetes mellitus. *J Clin Invest* 102:619–624, 1998