Extracellular Signal-Regulated Kinase Mediates Stimulation of TGF-β1 and Matrix by High Glucose in Mesangial Cells

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Abstract. High ambient glucose exerts its injurious effects on renal cells through nonenzymatic and enzymatic pathways, including altered signal transduction and upregulation of the transforming growth factor-β (TGF-β) system. Extracellular signal-regulated kinase (ERK), a member of the mitogen-activated protein kinase (MAPK) cascade, is activated in mesangial cells cultured in high glucose and in glomeruli of diabetic rats. However, the biologic consequences of ERK activation in the kidney have not been investigated. To clarify the role of ERK activation, mouse mesangial cells were exposed to normal (5.5 mM) or high (25 mM) glucose with or without addition of PD98059, a specific inhibitor of MAPK/ERK kinase (MEK), an upstream kinase activator of ERK. Cells that were exposed to high glucose exhibited significant increases in ERK activity, TGF-β1 expression (total protein, mRNA levels, and promoter activity), [^{3}H]-proline uptake, and α1(I) collagen and fibronectin mRNA levels. Treatment with PD98059 (up to 25 μM) significantly inhibited these parameters. In contrast, 25 μM PD98059 had no significant effect on any of the parameters measured in cells that were exposed to normal glucose. Overexpression of MAPK phosphatase CL100 prevented TGF-β1 promoter activation by high glucose, confirming the involvement of the MEK-ERK pathway in response to high glucose. The conclusion is that activation of ERK in mesangial cells is responsible for high-glucose–induced stimulation of TGF-β1 and contributes to the increased extracellular matrix expression.

Diabetic nephropathy is characterized by renal hypertrophy and accumulation of extracellular matrix (1). Many studies have demonstrated that various renal cell types cultured in high-glucose media exhibit the typical features of cellular hypertrophy and excessive production of extracellular matrix that are characteristic of diabetic nephropathy *in vivo* (2–4). It has been reported that high ambient glucose exerts its injurious effects through nonenzymatic and enzymatic pathways, including modulation of key signal transduction pathways and upregulation of the hypertrophic and profibrogenic cytokine transforming growth factor-β (TGF-β) (5). Of relevance to cellular signaling pathways, high ambient glucose increases *de novo* synthesis of diacylglycerol with subsequent activation of protein kinase C (PKC) (6,7). The activation of PKC has been proposed to be an important intracellular mediator of diabetic complications; the use of nonspecific and specific inhibitors of PKC isozymes has been shown to prevent alterations of cellular functions induced by high glucose in *in vitro* and *in vivo* models of diabetic kidney disease (8–11).

Recently, three mitogen-activated protein kinase (MAPK) families have been identified and characterized (12); these are extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), and p38 MAP kinase. These kinases can be activated by various extracellular stimuli, including growth factors and environmental stresses, and they play an essential role in the signal transduction cascades that lead to alterations in cell growth and other key functions (12,13). With regard to the diabetic state, it has been reported that ERK is activated in glomeruli of diabetic rats as well as in mesangial cells cultured under high-glucose conditions (14,15). ERK activation in these cells is believed to occur through a PKC-dependent mechanism (14). A recent report also demonstrated that p38 MAP kinase was activated by relatively high concentrations of extracellular glucose in various types of cells, including mesangial cells (16). In contrast to these two kinases, JNK is not activated in the glomeruli of diabetic rats or stimulated in mesangial cells upon exposure to high glucose (17). Thus, the activation of the ERK pathway could be involved in the intracellular signaling cascade that leads to cellular dysfunction in diabetes mellitus. It is likely that the activation of ERK is involved in regulating cellular functions, including proliferation and protein synthesis. For example, ERK can activate Elk-1, a member of the ternary complex factor, resulting in *c-fos* transcription and the subsequent formation of the activator protein-1 (AP-1) transcription factor complex (18,19). However, the biologic consequences of ERK activation in mesangial cells by high ambient glucose have not been explored.

We postulated that increased renal TGF-β bioactivity by high ambient glucose is important in the pathogenesis of dia-

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betic nephropathy (4,20–22). Studies that have used neutralizing anti–TGF-β antibodies have provided convincing evidence that the prosclerotic and hypertrophic effects of high glucose are largely mediated by the autocrine production and activation of TGF-β in glomerular mesangial cells, proximal tubular cells, and interstitial fibroblasts (2,4,23,24). We recently reported that high glucose or phorbol esters stimulate TGF-β1 production in mouse mesangial cells through a transcriptional mechanism (25). The TGF-β1 promoter has multiple AP-1–like consensus sites that respond to phorbol-ester/PKC stimulation (26). From these observations, it can be hypothesized that the activation of the PKC-ERK pathway by high glucose could be responsible for the induction of TGF-β1 and the production of extracellular matrix. To test this hypothesis, we examined in the current study the effect of PD98059, a specific inhibitor of MAPK/ERK kinase (MEK), on the expression of TGF-β1, α1(I) collagen, and fibronectin in mesangial cells that were cultured in high glucose. To confirm the involvement of the MEK-ERK pathway, we also assessed TGF-β1 promoter activity in response to high glucose after the overexpression of CL100, a MAP kinase phosphatase.

Materials and Methods

Cell Culture

Murine mesangial cells (MMC) were isolated and transformed with non–capsid-forming SV-40 virus to establish a permanent cell line with a stable, differentiated phenotype (24,27). For comparative purposes, untransformed mesangial cells obtained from kidneys of 10-wk-old SJL/J(H-2 s) mice (27) were also studied to asses the effects of high-glucose media on ERK activity. Cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies BRL, Gaithersburg, MD) containing a normal D-glucose concentration of 5.5 mM, 10% fetal bovine serum, 100 μg/ml streptomycin, 100 U/ml penicillin, and 2 mM glutamine. At 70% confluence, mesangial cells were cultured in fresh Dulbecco's modified Eagle's medium containing 2% fetal bovine serum with either 5.5 or 25 mM D-glucose for different time periods. In some experiments, cells were exposed to various concentrations of PD98059 or the general PKC inhibitor calphostin C (Calbiochem, La Jolla, CA).

ERK In Vitro Kinase Assay

Cells were lysed in a buffer containing 25 mM Tris-HCl (pH 7.4), 25 mM NaCl, 80 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 1 mM Na_3VO_4, 10 mM NaF, 4 mM ethyleneglycol-bis(β aminoethyl ether)-*N*,*N*′-tetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 1% Triton X-100. The cell lysates were centrifuged at 14,000 × *g* for 20 min, and protein concentrations were measured by a protein assay kit (Bio Rad, Richmond, CA). Cell lysates (400 μg) were incubated with 2 μg anti-ERK2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at 4°C. The immunoprecipitates were recovered by incubating with protein G sepharose (Amersham Pharmacia, Piscataway, NJ) for 1 h at 4°C. After centrifuging and washing three times with cell lysis buffer and once with kinase buffer (20 mM Tris-HCl [pH 7.4], 10 mM $MgCl_2$, 1 mM DTT, 1 mM ethyleneglycol-bis(β aminoethyl ether)-*N*,*N*′-tetraacetic acid, and 1 μM protein kinase inhibitor), the immunoprecipitates were incubated with 30 μl of a kinase buffer containing 20 μg of myelin basic protein (MBP), 50 μM adenosine triphosphate (ATP) and 2 μCi γ-[^{32}P]ATP for 15 min at 25°C. The mixture (15 μl) was spotted onto P-81 phosphocellulose paper (Whatman, Clifton, NJ), washed four times in 0.5% phosphoric acid, and rinsed with 95% ethanol. Phosphorylation activity was determined by a liquid scintillation counter.

Enzyme-Linked Immunosorbent Assay

Cell supernatants were frozen at −20°C until assayed by a TGF-β1 enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's specifications (R & D systems, Minneapolis, MN). In brief, the supernatants were activated with 1 N HCl followed by neutralization with 1.2 N NaOH/0.5 M HEPES to measure total TGF-β1. Samples were plated on anti–TGF-β type II receptor-coated microtiter plates and incubated for 3 h at room temperature. After vigorous washing, wells were incubated with anti–TGF-β1 antibody conjugated to horseradish peroxidase for 1.5 h, and substrate solution was added. The reaction was stopped by adding stop solution, and absorbance at 450 nm was measured. Total TGF-β1 protein production was reported per total cell protein content.

Immunoblot Analysis

Cell lysates were prepared in Laemmli sample buffer and boiled for 5 min. Aliquots (30 μg) were subjected to sodium dodecyl sulfate (SDS)-10% polyacrylamide gel and transferred to nitrocellulose membrane (Micron Separations Inc., Westborough, MA). The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline-0.1% Tween 20 at 4°C overnight and then probed with either anti-phospho-p44/42 antibody (New England Biolabs, Beverly, MA) or anti-MAP kinase phosphatase (MKP)-1 antibody (Santa Cruz Biotechnology) at room temperature for 3 h. A horseradish peroxidase-conjugated second antibody was used to allow the detection of immunoreactive bands using the enhanced chemiluminescence detection system (Amersham Pharmacia). For the assessment of protein amount of ERK, the membrane was reprobed with anti-ERK2 antibody (Santa Cruz Biotechnology).

Northern Blot Analysis

Murine TGF-β1, fibronectin, and α1(I) cDNA probes were synthesized by PCR and cloned into the pCRII TA cloning vector (Invitrogen, La Jolla, CA) as described previously (28). Total RNA was isolated using TRIzol reagent (Life Technologies BRL). For northern blots, 20 μg total RNA was electrophoresed through a 1.2% agarose gel with 0.67 M formaldehyde. The RNA was blotted onto nylon membrane (NEN Research Products, Boston, MA) by the capillary method and UV cross-linked. Membranes were prehybridized for 1 h at 65°C in a Church buffer containing 500 mM NaPO4 (pH 7.0), 5% SDS, 1% bovine serum albumin, and 1 mM ethylenediaminetetraacetate. The cDNA was labeled with [^{32}P]deoxycytidine 5'-triphosphate (Amersham Pharmacia) using a DNA labeling kit (Amersham Pharmacia). The membrane was hybridized in Church buffer for 16 h at 65°C in a rotating oven and was washed twice in 2X SSC (20X SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7.0) for 10 min each at room temperature, then in 2X SSC with 1% SDS for 15 min at 65°C, followed by two 15-min high-stringency washes in 0.1% SSC, 0.1% SDS at 65°C, if necessary. The membrane was autoradiographed with intensifying screens at −70°C (Kodak, Wilmington, DE). The blots were stripped and subsequently rehybridized with probes encoding mouse ribosomal protein L32 (mrpL32) (29) to account for small loading and transfer variations. Exposed films were scanned and analyzed with the NIH Image 1.62 program, and RNA levels relative to those of mrpL32 were calculated.

Plasmids, Transfection, and Luciferase Assay

Murine TGF-β1 promoter-reporter chimeric constructs were kindly provided by Dr. Andrew G. Geiser (30). The construct pA835 used contains −835 bp 5' from the A transcription start site of the murine TGF-β1 gene (30). The luciferase reporter gene used was cloned into the *Hin*dIII-*Kpn*I site of the pXP2 vector (31), which contains the luciferase gene without regulatory elements. The MAP kinase phosphatase CL100 in the expression plasmid pSG5 (Stratagene, La Jolla, CA) was kindly provided by Dr. Stephen M. Keyse (32,33). The β-galactosidase-containing plasmid pCH110 (Amersham Pharmacia) was used to control for transfection efficiency. MMC (50% confluent) were transfected by the Fugene 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN) following the manufacturer's instructions. After 16 h of transfection, cells were incubated in either normal or high glucose for an additional 24 h. Cells were harvested in reporter lysis buffer (Promega, Madison, WI). Luciferase and β-galactosidase assays were performed with reagents from Promega. Luciferase activity was normalized to β-galactosidase activity.

Measurement of [^{3}H]-Proline Incorporation

MMC were plated into 24-well plates (Nunclon, Roskilde, Denmark), and the media were changed the next day so that cells could be exposed for another 72 h to either 5.5 or 25 mM glucose with or without PD98059. For the last 16 h, cells were pulsed with 1 μCi [^{3}H]-proline (L-(2,3,4,5)-[^{3}H]-proline, Amersham Pharmacia). Radiolabeled MMC were washed twice in ice-cold phosphate-buffered saline and then precipitated twice in ice-cold 10% TCA, redissolved in 0.5 ml of 0.5 N NaOH with 0.1% Triton X-100. After neutralization with 0.5 N HCl, the incorporated radioactivity was counted in a liquid scintillation counter. Proline incorporation was corrected for the cellular protein content.

Statistical Analyses

Data are presented as mean ± SEM. ANOVA followed by Scheffe's test was used for multiple comparisons. Two groups were compared by unpaired *t* test. $P < 0.05$ was considered significant.

Results

Activation of ERK by High Glucose in Mouse Mesangial Cells

We first measured the activity of ERK in MMC. Cells were exposed to either normal (5.5 mM) or high glucose (25 mM) for 24 and 72 h. ERK activity was analyzed by both *in vitro* kinase assay and immunoblot analysis. As shown in Figure 1A, the activity of ERK was significantly increased in MMC by exposure to high glucose for 24 and 72 h. Similar results were obtained in untransformed mouse mesangial cells: exposure of these cells to high glucose for 72 h significantly increased ERK activity (1.61 ± 0.14 pmol/min per mg protein) as compared with normal glucose (1.20 ± 0.09, $n = 5$, $P < 0.05$). The immunoblot analysis using anti-phospho ERK antibody, which recognizes phosphorylated threonine 202 and tyrosine 204 of p44/p42 ERK, detected enhanced phosphorylation of p44/42 ERK by high glucose (Figure 1, B and C). This result suggests that MEK, upstream of ERK, is also activated by high glucose. The activation of ERK by high glucose was probably PKC-dependent because the addition of calphostin C, a PKC inhibitor, prevented the activation of ERK (Figure 1D). Consistent with a previous report (14), our results demonstrate that the ERK cascade may be activated through a PKC-dependent pathway in mouse mesangial cells cultured under high-glucose conditions. We also confirmed the ability of PD98059, a known specific inhibitor of MEK (34), to inhibit the activation of ERK in mesangial cells. Addition of 25 μM PD98059 to MMC completely inhibited the activation of ERK by high glucose (Figure 1, B and C).

Effect of PD98059 on High-Glucose–Induced TGF-β1 Protein Production

To assess the biologic significance of ERK activation, we first examined whether PD98059 could inhibit TGF-β1 production by high glucose. MMC were cultured in media containing normal and high glucose with various concentrations of PD98059 for 72 h. TGF-β1 protein in the supernatant was measured by ELISA. As we reported previously (25), total TGF-β1 production was significantly increased in supernatants of cells cultured in high glucose compared with cells cultured in normal glucose. TGF-β1 protein production was inhibited by exposure to PD98059 in a dose-dependent manner. Twenty-five μM PD98059 completely prevented the increase in TGF-β1 by high glucose without changing TGF-β1 production in cells cultured in normal glucose (Figure 2).

Effect of PD98059 on High-Glucose–Induced TGF-β1 Gene Expression

To confirm the inhibitory effect of PD98059 on TGF-β1 production, we examined the high-glucose–induced TGF-β1 gene expression by Northern blot analysis and promoter assay. TGF-β1 mRNA was significantly increased in cells cultured in high glucose, and PD98059 was able to inhibit completely the increase in TGF-β1 mRNA (Figure 3). We next evaluated the effect of PD98059 on TGF-β1 promoter activity. Because we recently demonstrated that only the pA835 promoter construct, which contains 835 bp upstream from the transcription start site of the murine TGF-β1, exhibited significant activation by high glucose (25), we used in this experiment the pLA835 plasmid, which contains the same region of TGF-β1 promoter fused to the luciferase gene. MMC were transiently cotransfected with pLA835 and the β-galactosidase-containing plasmid pCH110, to control for transfection efficiency. Cells were then cultured in high-glucose media with or without PD98059 for an additional 24 h. Relative luciferase activity to β-galactosidase was calculated. As shown in Figure 4, high glucose significantly stimulated promoter activity of TGF-β1, whereas PD98059 inhibited the induction of TGF-β1 promoter activity. Treatment of mesangial cells cultured in normal glucose media with 25 μM PD98059 affected neither baseline mRNA expression nor promoter activity.

Effect of Overexpression of MAP Kinase Phosphatase CL100 on TGF-β1 Promoter Activity

To confirm further the involvement of ERK in TGF-β1 induction under high-glucose conditions, we cotransfected cells with a plasmid expressing MAP kinase phosphatase CL100, which inactivates ERK (32,33). Cotransfection of MMC with pSG5-CL100 prevented the induction of TGF-β1

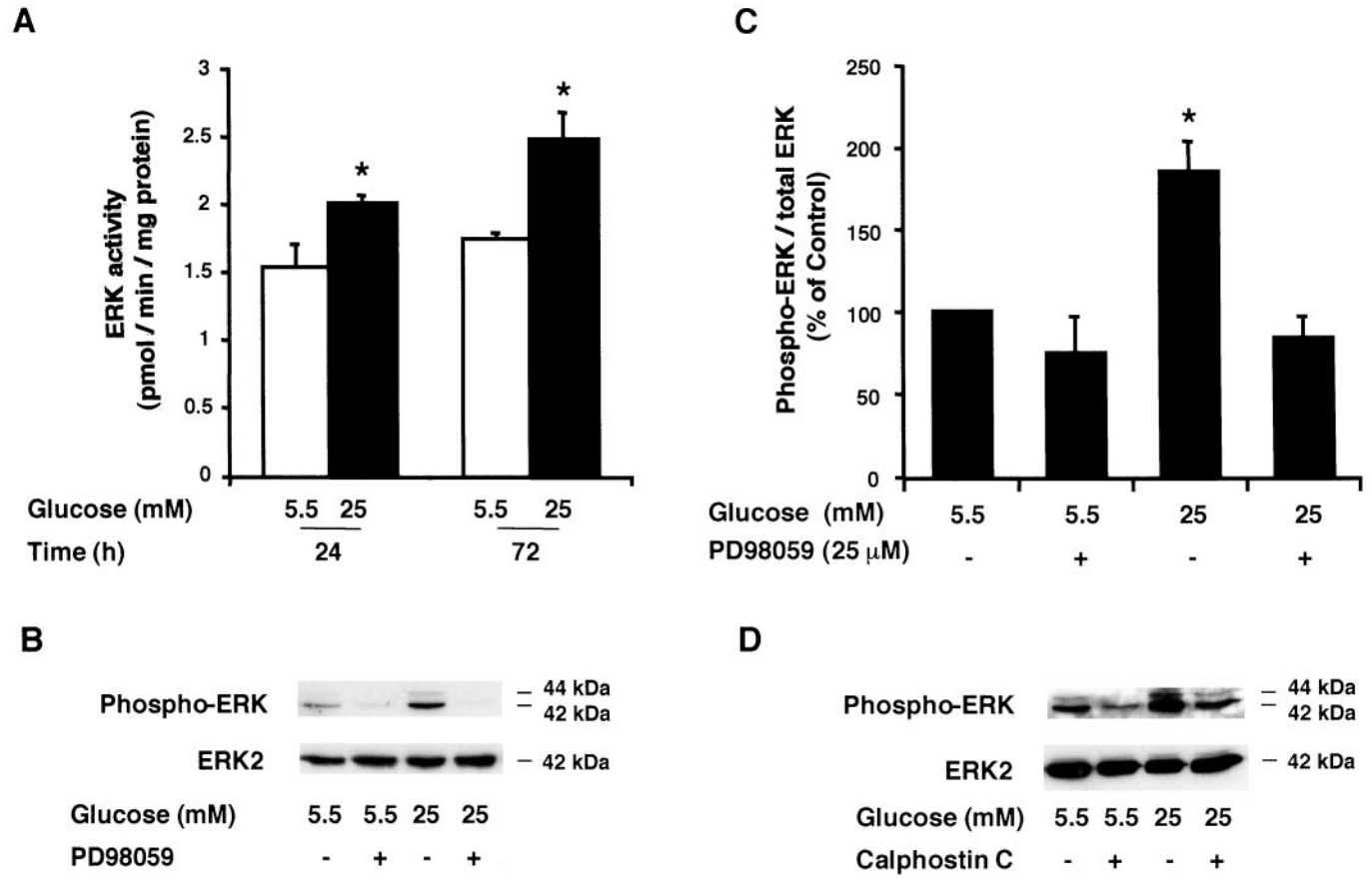

Figure 1. Activation of extracellular signal-regulated kinase (ERK) in mouse mesangial cells (MMC) cultured in high glucose. (A) MMC were cultured in normal (5.5 mM) or high glucose (25 mM) media for 24 or 72 h. The activity of ERK was measured by *in vitro* kinase assay. Results are mean ± SEM, $n = 4$ or 5. *, $P < 0.05$ *versus* normal glucose. (B) Cells were cultured in normal or high glucose media for 72 h with or without PD98059 (25 μM). ERK activity (phosphorylation) and ERK protein content were analyzed by immunoblotting using anti-phospho-ERK antibody (upper band) and anti-ERK2 antibody (lower band), respectively. (C) Quantitative results of phosphorylation of ERK; mean ± SEM, $n = 5$. *, $P < 0.05$ *versus* other groups. (D) Cells were cultured in normal or high glucose for 72 h with or without calphostin C (1 μM) for the last 4 h. The phosphorylation of ERK and ERK protein content were analyzed by immunoblotting, as above.

promoter by high glucose; however, expression of the empty, control vector produced the expected increment in TGF-β1 promoter activity in high-glucose media (Figure 5A). The protein content of MAP kinase phosphatase and ERK were not changed in cells transfected with CL100 (Figure 5B). We also confirmed that the overexpression of CL100 in the cells prevented the activation of ERK by high glucose (Figure 5C).

Effect of PD98059 on High-Glucose–Induced Matrix Production

We next assessed whether PD98059 could inhibit extracellular matrix production induced by high glucose. We examined [^{3}H]-proline incorporation and performed Northern blot analysis for the expression of α1(I) collagen and fibronectin. Figure 6 shows that [^{3}H]-proline incorporation into MMC was significantly increased in cells grown in high-glucose media, as was expected (24). The incubation with PD98059 significantly inhibited the increment in [^{3}H]-proline incorporation into MMC. Northern blot analyses (Figure 7) demonstrated that the mRNA for α1(I) collagen and fibronectin were increased by approximately 50% in high-glucose media and that PD98059 abolished these increases without effecting mRNA expression in cells cultured in normal glucose.

Discussion

Many studies have demonstrated that various metabolic mediators and signaling pathways are activated when kidney cells are exposed to high ambient glucose. These include the polyol pathway (35), *de novo* synthesis of diacylglycerol with stimulation of PKC (6), and activation of the hexosamine pathway (36). Although the involvement of PKC activation in the pathogenesis of diabetic complications has been well documented (37), the role of other protein kinases in the functional abnormalities of the diabetic state has not been fully established. Recently, Haneda *et al.* (14) reported that MEK and ERK are activated in glomeruli of streptozotocin-induced diabetic rats and in rat mesangial cells cultured in high-glucose media and that the activation of this cascade in mesangial cells can be attenuated by the PKC inhibitor calphostin C. In accord with these findings, we show in this study that the ERK cascade is

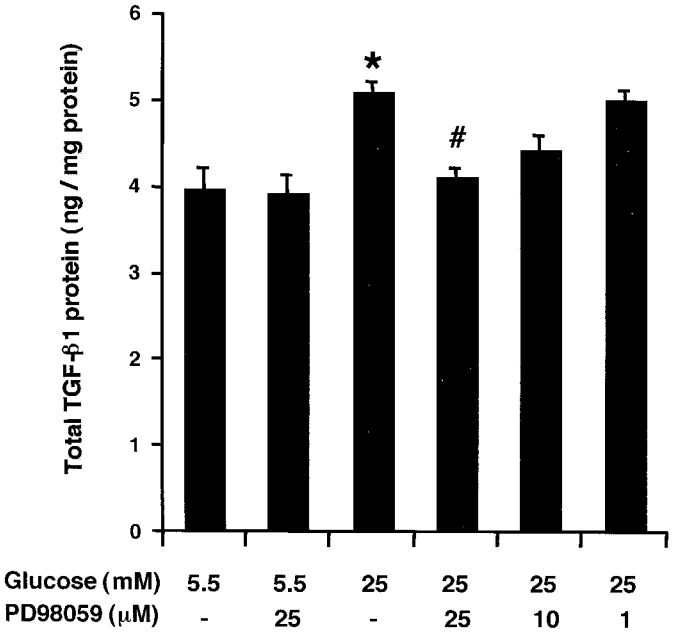

Figure 2. The effect of PD98059 on high-glucose–induced transforming growth factor-β1 (TGF-β1) protein production. MMC were cultured in normal- and high-glucose media with different concentrations of PD98059 for 72 h. The supernatants were activated with 1 N HCl and neutralized, and total TGF-β1 protein was measured by enzyme-linked immunosorbent assay. Results are mean ± SEM, $n = 4$. *, $P < 0.05$ *versus* normal glucose; #, $P < 0.05$ *versus* high glucose without PD98059.

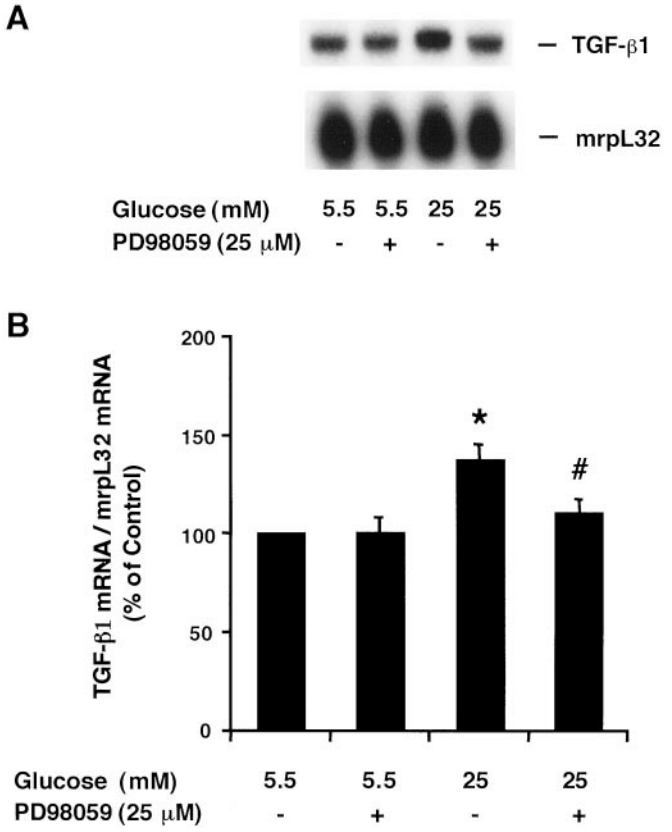

Figure 3. The effect of PD98059 on high-glucose–increased TGF-β1 mRNA. MMC were cultured in normal- and high-glucose media with PD98059 (25 μM) for 48 h. (A) Representative Northern blot hybridized with TGF-β1 followed by mouse ribosomal protein L32 (mrpL32) to control for RNA loading and transfer. (B) Quantitative results of TGF-β1/mrpL32 mRNA ratios; mean ± SEM, $n = 5$. *, $P < 0.05$ *versus* other groups; #, $P < 0.05$ *versus* high glucose without PD98059.

activated in mouse mesangial cells grown in high-glucose media. Immunoblot analysis using anti-phospho-ERK antibody and *in vitro* kinase assay using MBP as substrate demonstrated increased phosphorylation of ERK and MBP, respectively. With the use of PD98059, a specific inhibitor of the ERK kinase MEK (34), we extended these studies by showing that ERK activation in mesangial cells is required for the induction of TGF-β1 and the stimulation of fibronectin and type I collagen by high ambient glucose.

We previously reported that high glucose stimulates TGF-β1 production in mouse mesangial cells through a transcriptional mechanism that involves a specific glucose-responsive DNA-binding element (25). We also recently reported that high glucose stimulates the TGF-β type II receptor in mouse mesangial cells (22). In this study, we show that treatment of mesangial cells with PD98059 significantly inhibits the high-glucose–induced TGF-β1 protein production, mRNA level, and promoter activity. Furthermore, and consistent with the requirement for ERK phosphorylation in the stimulation of TGF-β1 by high glucose, we show that the overexpression of CL100, a MAPK phosphatase, prevents the activation of the TGF-β1 promoter by high glucose.

ERK activation is able to activate Elk-1, a member of the ternary complex factors that enhance the expression of *c-fos* (18) and the subsequent DNA binding of the transcription factor AP-1 (19). The TGF-β1 promoter has multiple AP-1–like consensus sites that respond to phorbol-ester/PKC stimulation (26). An increase in AP-1 binding has been reported to mediate the regulation of the TGF-β1 gene (38). Thus, it is likely that the stimulation of ERK by high glucose transduces a signal to the nucleus, where it regulates the expression of certain target genes through AP-1 transactivation (18,19). In fact, it has been reported that high glucose stimulates the expression of *c-fos* and *c-jun*, components of the AP-1 complex (39), and the binding of AP-1 to DNA in cultured mesangial cells (40). Together, these observations suggest that the ERK pathway plays an important role in the transcriptional activation of TGF-β1 in mesangial cells cultured under high-glucose conditions. Other studies have suggested that the ERK pathway is itself also involved in the signaling events downstream of the TGF-β stimulus (41,42).

We also provide evidence that the activation of ERK by high glucose is required for the production of extracellular matrix proteins, including α1(I) collagen and fibronectin. Treatment with PD98059 prevents the high-glucose–induced uptake of proline, a major constituent of collagen proteins, and the increased mRNA levels of α1(I) collagen and fibronectin. There are two possible mechanisms for these inhibitory effects of

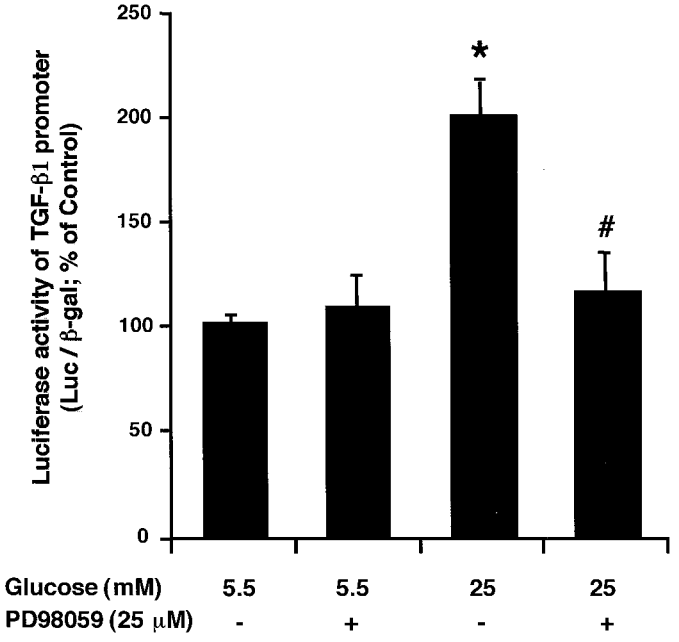

Figure 4. The effect of PD98059 on high-glucose–stimulated TGF-β1 promoter activity. MMC were transiently cotransfected with 1 μg of pLA835, the TGF-β1 promoter-luciferase construct, and 1 μg of pCH110, the β-galactosidase-containing construct. Cells were then incubated in normal- or high-glucose media with or without PD98059 (25 μM) for 24 h. Luciferase and β-galactosidase assays were performed. Results are mean ± SEM, $n = 4$. *, $P < 0.01$ *versus* other groups; #, $P < 0.01$ *versus* high glucose without PD98059.

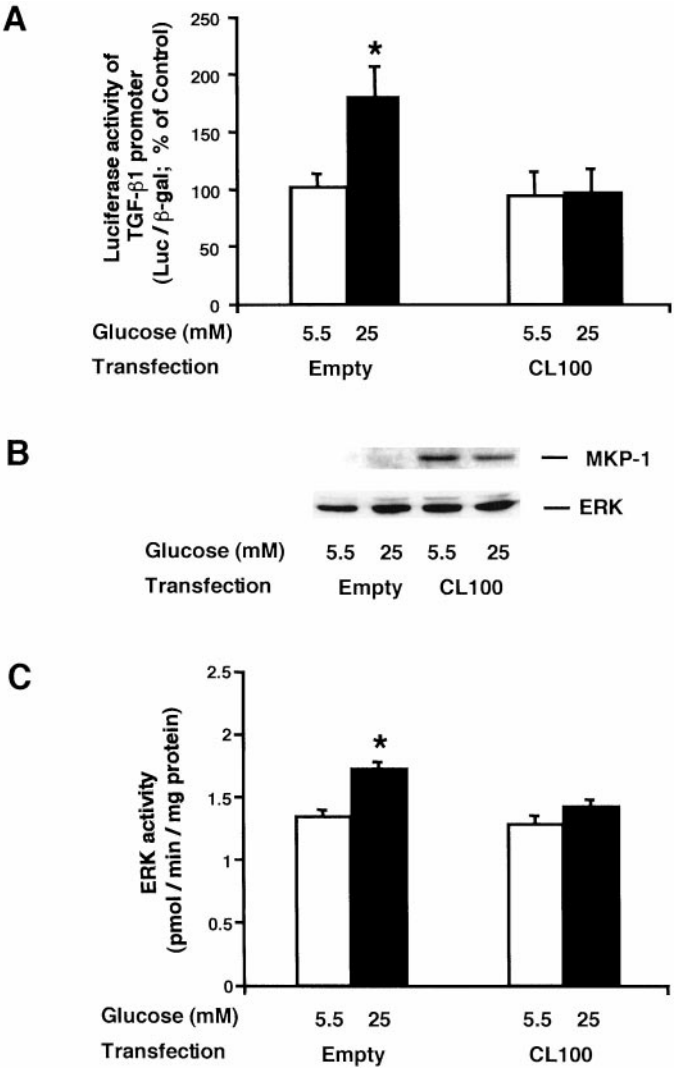

Figure 5. The effect of overexpression of MAP kinase phosphatase (MKP-1) on TGF-β1 promoter activity and ERK activity. MMC were transiently cotransfected with 1 μg of pLA835, 1 μg of pCH110, and 2 μg of either pSG5-CL100 (CL100), the MKP-1 expression vector, or the pSG5 empty vector (empty). Cells were then incubated in normal- or high-glucose media for 24 h. (A) Luciferase and β-galactosidase assays were performed. Results are mean ± SEM, $n = 4$. *, $P < 0.01$ *versus* other groups. (B) The expression of CL100 and ERK were analyzed by immunoblotting using anti–MKP-1 and anti-ERK2 antibodies, respectively. (C) The activity of ERK was measured by *in vitro* kinase assay. Results are mean ± SEM, $n = 6$. *, $P < 0.05$ *versus* other groups.

PD98059 on matrix expression. First, PD98059 may directly inhibit the production of extracellular matrix components. Although several reports incriminate PKC activation in renal matrix production in diabetes (8,9,11), little is known about the direct contribution of ERK activation on matrix protein synthesis. Fibronectin gene expression can be regulated through the binding of AP-1 to a cAMP response element in the promoter region (43). A recent report has demonstrated that PD98059 inhibits the mechanical stretch-induced fibronectin production through the inhibition of the ERK–AP-1 pathway (44). Furthermore, expression of TGF-β–induced type I collagen is mediated via the ERK pathway in certain cell types, including mesangial cells (41,42). Together, these observations strongly suggest that ERK activation may be directly involved in the regulation of extracellular matrix protein gene expression, at least for type I collagen and fibronectin.

The second possibility is that the high-glucose–induced matrix production is inhibited by PD98059 through the inhibition of the profibrotic cytokine TGF-β1. This possibility is supported by our previous report demonstrating that treatment of mesangial cells with neutralizing anti–TGF-β antibodies attenuates high-glucose–induced proline uptake and the synthesis of types I and IV collagen (24). Furthermore, inhibition of TGF-β bioactivity in diabetic mice using either neutralizing anti–TGF-β antibodies (28,45) or antisense TGF-β1 oligodeoxynucleotides (46,47) is capable of reversing the renal hypertrophy and upregulation of extracellular matrix expression. Together, these results suggest that inhibition of TGF-β1 is one of the important approaches that can be considered for the prevention of the functional and structural consequences of diabetic renal disease.

In addition to hyperglycemia, many factors in the diabetic state, such as glomerular hypertension, oxidative stress, nonenzymatic glycation adducts, and growth factors, have been proposed to mediate diabetic nephropathy (5). Some of these factors, such as mechanical stretch (44), angiotensin II (48), endothelin (49), and advanced glycation end products (50), are

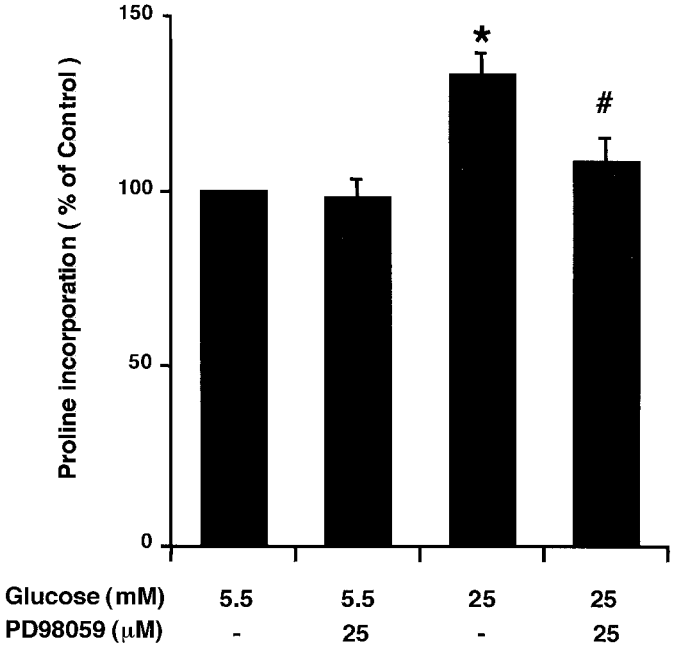

Figure 6. The effect of PD98059 on high-glucose–induced proline incorporation. MMC were cultured in normal- or high-glucose media with or without PD98059 (25 μM) for 72 h. For the last 16 h, cells were pulsed with [^{3}H]-proline. [^{3}H]-proline incorporation was corrected by cell protein content. Results are mean ± SEM, $n = 6$. *, $P < 0.05$ *versus* normal glucose; #, $P < 0.05$ *versus* high glucose without PD98059.

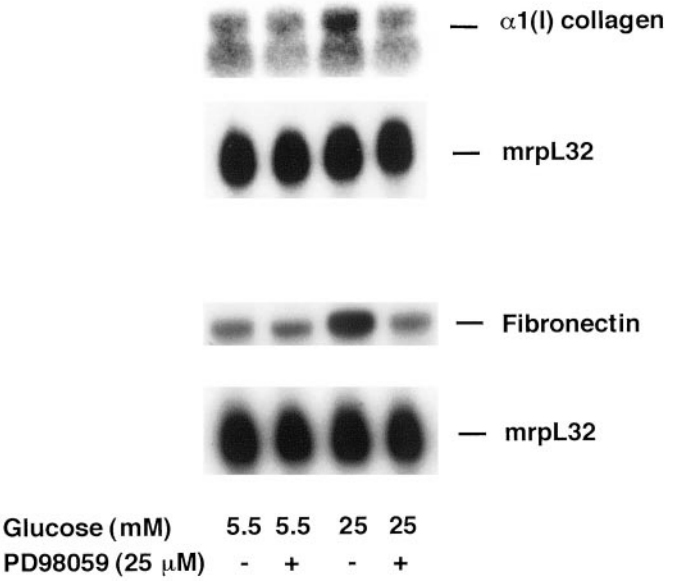

Figure 7. The effect of PD98059 on high-glucose–induced extracellular matrix expression. MMC were cultured in normal- and high-glucose media with or without PD98059 (25 μM) for 48 h. Representative Northern blots, of three different experiments, hybridized with α1(I) collagen and fibronectin, followed by mrpL32 as control ($n = 3$). High-glucose media increased α1(I) collagen and fibronectin mRNA levels, and PD98059 treatment abolished these increases without affecting their levels in normal-glucose media.

able to activate ERK in cultured renal cells. These same factors are also activators of the TGF-β system in the kidney (5). From all of these observations, we can conclude that the activation of ERK by high ambient glucose and other factors leads to increased TGF-β1 production as well as extracellular matrix proteins. Renal overproduction of TGF-β1, in turn, can further exaggerate the excess synthesis and accumulation of extracellular matrix proteins in the diabetic milieu (Figure 8). Thus, interventions at the level of ERK to intercept this intracellular signaling pathway may be a useful approach for inhibiting not only the TGF-β system but also other pertinent downstream events that are characteristic of diabetic renal disease, such as increased matrix expression.

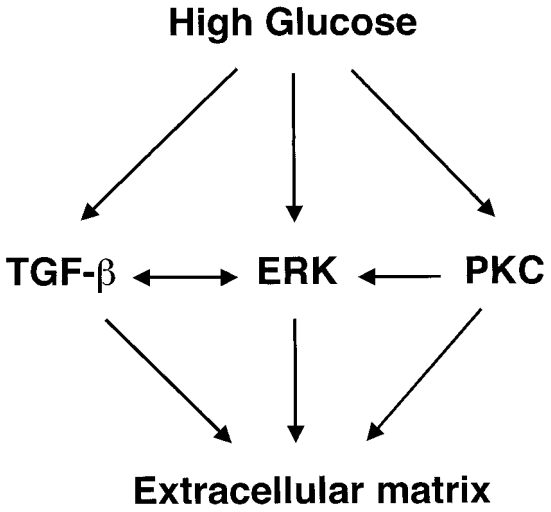

Figure 8. Summary diagram depicting the signaling pathways for high-glucose–stimulated extracellular matrix production in mesangial cells.

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