Stretch-Induced Overproduction of Fibronectin in Mesangial Cells Is Mediated by the Activation of Mitogen-Activated Protein Kinase

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An excessive production of extracellular matrix (ECM) proteins in glomerular mesangial cells is considered to be responsible for the development of mesangial expansion seen in diabetic nephropathy. Mechanical stretch due to glomerular hypertension has been proposed as one of the factors leading to an increase in the production of ECM proteins in mesangial cells, but the precise mechanism of stretch-induced overproduction of ECM proteins has not been elucidated. Herein, we provide the evidence that mitogen-activated protein kinase (MAPK) may play a key role in the overproduction of fibronectin (FN) in mesangial cells exposed to mechanical stretch. MAPK, also termed extracellular signal–regulated kinase (ERK) and c-Jun NH_2-terminal kinase (JNK), was activated by mechanical stretch in time- and intensity-dependent manners. Stretch-induced activation of ERK was inhibited by herbimycin A, a tyrosine kinase inhibitor, but not by GF109203X or calphostin C, the inhibitors of protein kinase C. Mechanical stretch also enhanced DNA-binding activity of AP-1, and this enhancement was inhibited by PD98059, an inhibitor of MAPK or ERK kinase (MEK). Furthermore, mechanical stretch stimulated the expression of FN mRNA followed by a significant increase in its protein accumulation. PD98059 could prevent stretch-induced increase in the expression of FN mRNA and protein. These results indicate that the activation of ERK may mediate the overproduction of ECM proteins in mesangial cells exposed to mechanical stretch, an in vitro model for glomerular hypertension seen in diabetes. *Diabetes* 48:595–602, 1999

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BSA, bovine serum albumin; CRE, cAMP-responsive element; DTT, dithiothreitol; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal–regulated kinase; FN, fibronectin; JNK, c-Jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MEK, MAPK or ERK kinase; PBS, phosphate-buffered saline; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; PTK, protein tyrosine kinase; TGF-β1, transforming growth factor-β1.

The expansion of the glomerular mesangium is one of the major histologic characteristics of diabetic nephropathy (1,2). The degree of mesangial expansion was found to be strongly related to clinical manifestations of diabetic nephropathy, such as albuminuria and a decrease in glomerular filtration rates (2). This mesangial expansion is caused by an excessive accumulation of extracellular matrix (ECM) proteins, such as type IV collagen and fibronectin (FN), in glomerular mesangium. Since glomerular mesangial cells are the cells responsible for the production of these mesangial matrix proteins (3,4), it is important to elucidate the mechanisms of an excessive production of ECM proteins by mesangial cells under the diabetic milieu. Recent reports suggest that mesangial cells exposed to high concentrations of glucose are able to produce excessive amount of ECM proteins (5–7). Intracellular metabolic abnormalities, such as the activation of polyol pathway (8,9), an increase in de novo synthesis of diacylglycerol followed by the activation of protein kinase C (PKC) (10–16), and an induction of transforming growth factor-β1 (TGF-β1) (17–19) have been suggested as mechanisms responsible for an excessive production of ECM proteins by mesangial cells exposed to high concentrations of glucose.

In addition, glomerular hypertension due to intrarenal hemodynamic changes in diabetes has been reported to contribute to the development and progression of diabetic nephropathy (20,21). An increase in glomerular pressure has been recently postulated to cause mechanical stretch of mesangial cells (22,23), and an excessive production of ECM proteins was found to be induced in mesangial cells exposed to mechanical stretch (23–27). Although mechanical stretch was shown to activate both PKC (27,28) and protein tyrosine kinase (PTK) (26) in mesangial cells, the precise intracellular mechanisms of stretch-induced overproduction of ECM proteins have not been fully elucidated.

Recent evidence suggests that the mitogen-activated protein kinase (MAPK) family, including extracellular signal–regulated kinase (ERK) and c-Jun NH_2-terminal kinase (JNK), plays an important role in the intracellular signal transduction system leading to various cellular functions (29–31). ERK was found to phosphorylate and activate Elk-1, one of the ternary complex factors, which could enhance the expression of c-*fos* (32), leading to an enhancement of DNA binding activity of AP-1 (33–35). We and others have reported that ERK is activated in mesangial cells by stimuli which activate PKC and PTK (36,37). We have also reported that ERK is activated

through a PKC-dependent mechanism in mesangial cells cultured under high-glucose conditions (38). From these observations, we hypothesized that mechanical stretch might activate ERK through either a PKC- or a PTK-dependent mechanism and thus enhance the production of ECM proteins in mesangial cells. To test this hypothesis, we measured the activities of ERK and its contribution to the production of FN in mesangial cells exposed to mechanical stretch.

RESEARCH DESIGN AND METHODS

Materials. Anti–active ERK antibody and consensus oligonucleotide of AP-1 were purchased from Promega (Madison, WI). Anti-ERK2 antibody was bought from Santa Cruz Biotechnology (Santa Cruz, CA). Rat plasma FN and rabbit anti-rat FN antibody were purchased from Chemicon (Temecula, CA). Bovine myelin basic protein (MBP) and herbimycin A were bought from Sigma (St. Louis, MO). PD98059 was bought from New England Bio-lab (Beverly, MA). GF109203X (bisindolylmaleimide I) and calphostin C were purchased from Calbiochem (La Jolla, CA) and Wako Pure Chemical Industries (Osaka, Japan), respectively. The glutathione-S-transferase (GST) fusion protein expression vector containing the transactivation domain of c-Jun (amino acids 1–79), pGEX2T-c-Jun, was a gift from Dr. Michael Karin (University of California, San Diego), and mouse TGF-β1 cDNA was provided by Dr. Fuad N. Ziyadeh (University of Pennsylvania, Philadelphia). [γ-^{32}P]ATP and [α-^{32}P]dCTP were bought from New England Nuclear (Boston, MA). All other reagents were of chemical grade and purchased from standard suppliers.

Experimental protocol. Mesangial cells were obtained from a culture of glomeruli isolated from male Sprague-Dawley rats weighing 100–150 g in RPMI 1640 medium containing 20% fetal bovine serum and antibiotics. Cultured cells were identified as mesangial cells, as previously described (8,39). Mesangial cells from the 3rd to 6th passages were plated on six-well plates with either flexible or rigid bottom coated with type I collagen (Flexcell, Mckeesport, PA) at a density of 5×10^4 cells/well. Subconfluent cells were made quiescent by incubating in RPMI 1640 medium containing 0.2% bovine serum albumin (BSA) for 24 h and subjected to cyclic stretch. Stretch was mediated by controlled cycles of vacuum applied to the underside of the flexible-bottom culture well using a computer-assisted system (Flexercell Strain Unit; Flexcell). In some experiments, cells were incubated with various concentrations of PD98059, an inhibitor of MAPK or ERK kinase (MEK) (40,41), or GF109203X, a PKC inhibitor (42), for 90 min; 0.1–10 μmol/l calphostin C for 4 h (38); or 1 μmol/l herbimycin A, a PTK inhibitor, for 16 h before the exposure to stretch stress.

Measurement of the activities of ERK and JNK. The activities of ERK were measured by in gel kinase assay (36,38,43) or by the immunoblot analysis of active ERK. For in gel kinase assay, mesangial cells were lysed with ice-cold lysis buffer (50 mmol/l Tris-HCl, pH 7.5, 1% Nonidet P-40, 140 mmol/l NaCl, 1 mmol/l Na_3VO_4, 50 mmol/l NaF, 1 mmol/l EGTA, 50 U/ml aprotinin, 1 mmol/l phenylmethylsulfonyl fluoride [PMSF], and 1 μg/ml leupeptin) and sonicated at 4°C for 10 s. Cell lysate was centrifuged at 12,000*g* at 4°C for 30 min, and the supernatant was used for kinase assay. The concentrations of the proteins in the supernatant were measured using a protein assay kit (Bio-Rad, Hercules, CA). Cell lysate (50 μg) was applied to 10% SDS-polyacrylamide gel containing 0.5 mg/ml MBP. After electrophoresis, SDS was removed from the gel by washing with buffer A (50 mmol/l HEPES, pH 7.4, and 5 mmol/l mercaptoethanol) containing 20% 2-propanol. Proteins in the gel were denatured in buffer A with 6 mol/l guanidine-HCl at room temperature for 1 h and renatured in buffer A with 0.04% Tween 40 at 4°C for 16 h. The gel was incubated in a buffer (25 mmol/l HEPES, pH 7.4, 10 mmol/l $MgCl_2$, 0.1 mmol/l EGTA, and 5 mmol/l 2-mercaptoethanol) containing 50 μmol/l ATP with 250 μCi [γ-^{32}P]ATP at 30°C for 1 h for kinase reaction. Then the gel was extensively washed with 5% trichloroacetic acid and 10 mmol/l sodium pyrophosphate and subjected to autoradiography. Radioactivity of the corresponding bands was measured quantitatively using storage phosphor imaging system (Molecular Analyst; Bio-Rad).

For the immunoblot analysis of active ERK, mesangial cells were lysed in 30 μl/well of cell lysis buffer (62.5 mmol/l Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 50 mmol/l dithiothreitol [DTT]) and sonicated at 4°C for 10 s. After boiling at 100°C for 5 min, samples (25 μg protein) were electrophoresed on SDS-PAGE (12% acrylamide gel), as described by Laemmli (44), and transferred to a polyvinylidene difluoride filter (Immobilon; Millipore, Marbolo, MA) using Multiphor II (Pharmacia LKB, Uppsala, Sweden). After blocking with 5% nonfat milk and 0.1% Tween 20 in phosphate-buffered saline (PBS) at 4°C overnight, the filter was incubated with anti–active ERK antibody (1:10,000 dilution) in a dilution buffer (PBS containing 5% BSA and 0.1% Tween 20) at room temperature for 3 h. The filter was washed three times with PBS containing 0.1% Tween 20 and then incubated with horseradish peroxidase–conjugated anti-rabbit IgG at room temperature for 1 h. The bands were detected using enhanced chemiluminescence (Amersham, Buckinghamshire, U.K.). The filter was reprobed with anti-ERK2 antibody.

The activities of JNK were evaluated by measuring its ability to phosphorylate GST-c-jun (45,46). Cells were lysed in a buffer containing 25 mmol/l HEPES, pH 7.5, 0.3 mol/l NaCl, 1.5 mmol/l $MgCl_2$, 0.2 mmol/l EDTA, 0.5 mmol/l DTT, 20 mmol/l β-glycerophosphate, 0.1% Triton X-100, 1 mmol/l PMSF, 20 μg/ml aprotinin, and 20 μg/ml leupeptin. The cell lysate (500 μg protein) was incubated with 20 μl of GST-c-Jun fusion protein bound to glutathione-sepharose beads at 4°C for 3 h. The beads were recovered by centrifugation and washed three times with a buffer containing 20 mmol/l HEPES, pH 7.7, 50 mmol/l NaCl, 2.5 mmol/l $MgCl_2$, 0.1 mmol/l EDTA, and 0.05% Triton X-100 and once with a kinase buffer (20 mmol/l HEPES, pH 7.6, 20 mmol/l $MgCl_2$, 20 mmol/l β-glycerophosphate, 20 mmol/l p-nitrophenyl phosphate, 0.1 mmol/l Na_3VO_4, 2 mmol/l DTT). The beads were incubated with 30 μl of a kinase buffer containing 20 μmol/l ATP with 5 μCi [γ-^{32}P]ATP at 30°C for 20 min. The reaction was terminated by adding 30 μl of 3× Laemmli sample buffer, and samples were boiled at 100°C for 5 min. Samples were resolved on 12% SDS-PAGE and subjected to autoradiography.

Measurement of DNA binding activities of AP-1. Nuclear extract was prepared as described by Sadowski and Gilman (47) with modifications (46). In brief, cells were lysed with hypotonic buffer (20 mmol/l HEPES, pH 7.9, 1 mmol/l EGTA, 1 mmol/l EDTA, 20 mmol/l NaF, 1 mmol/l Na_3VO_4, 1 mmol/l $Na_4P_2O_7$, 1 mmol/l DTT, 0.5 mmol/l PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin) with 0.6% Nonidet P-40 and centrifuged at 16,000*g* for 20 s. Pellets were resuspended in high-salt buffer (hypotonic buffer with 420 mmol/l NaCl and 20% glycerol), followed by rotating at 4°C for 30 min and centrifuging at 16,000*g* for 20 s. The supernatants were used as nuclear proteins for a gel mobility shift assay. Nuclear proteins (3 μg) were incubated in a binding buffer [20 mmol/l HEPES, pH 7.9, 1.8 mmol/l $MgCl_2$, 2 mmol/l DTT, 0.5 mmol/l EDTA, 0.5 mg/ml BSA, and 2 μg poly (dI-dC) · poly (dI-dC)] at room temperature for 30 min, with or without unlabeled AP-1 or NF-κB oligonucleotides. AP-1 consensus oligonucleotide end-labeled with [α-^{32}P]dCTP was added to the mixture, and the incubation was continued for another 20 min at room temperature. The reaction mixtures were electrophoresed through 4% low anionic polyacrylamide gel and subjected to autoradiography.

Northern blot analysis. Stretch-induced gene expression of FN and TGF-β1 was examined by Northern blot analysis as previously described (48). Total RNA (12 μg) isolated by a guanidinium and phenol extraction (TRIzol Reagent; Gibco BRL, Grand Island, NY) was separated by formaldehyde/1.0% agarose gel electrophoresis and transferred onto a nylon membrane. After immobilizing the RNA by heating the membrane for 2 h at 80°C, the membrane was hybridized with rat FN cDNA or mouse TGF-β1 cDNA labeled with [α-^{32}P]dCTP by a random primer method (BcaBEST; TAKARA, Shiga, Japan) in a buffer (0.5 mol/l $NaPO_4$, pH 7.0, 1% BSA, 7% SDS, and 1 mmol/l EDTA) at 65°C for 16 h. After radioactive probes were stripped off the membrane, the membrane was rehybridized with radioactive probes of acidic ribosomal phosphoprotein PO (36B4) as an internal standard (49).

Assessment of accumulation of FN. Accumulation of FN in the medium was assessed by inhibition enzyme-linked immunosorbent assay (ELISA) (6,7). In brief, 50 μl FN standard or sample was incubated with the same volume of polyclonal rabbit anti-rat FN antibody diluted with PBS containing 0.5% BSA at room temperature for 30 min. Ninety microliters reaction mixture was then transferred to the 96 wells of a multiwell plate (Falcon; Becton Dickinson, Lincoln Park, NJ) precoated with FN (200 ng FN/well in 0.02 mol/l carbonate buffer, pH 9.6) at 4°C overnight. The plate was placed at 37°C for 60 min. After each well was rinsed three times with a washing buffer (PBS with 0.1% Tween 20), 100 μl anti-rabbit IgG antibody conjugated with peroxidase (Tago, Burlingame, CA) was added to the well, incubated at 37°C for 60 min, and rinsed three times. Final reaction mixture (0.04 mg/dl *o*-phenylenediamine hydrochloride and 0.06% H_2O_2 in 0.05 mol/l citrate buffer, pH 5.0) was then added and incubated at room temperature for 2 min. The reaction was stopped by adding 50 μl H_2SO_4, and absorbance at 490 nm was measured. Detection limit was 0.15 μg/ml FN. Results were normalized by cellular DNA measured spectrofluorometrically using bisbenzimide (Hoechst 33258) (50).

Statistical analysis. Results were expressed as mean ± SD. Analysis of variance (ANOVA) with subsequent Scheffé's test was used to determine significant difference in multiple comparisons.

RESULTS

Activation of ERK and JNK in mesangial cells exposed to mechanical stretch. Because, in the previous studies, an overproduction of ECM proteins was shown in mesangial cells exposed to mechanical stretch provided at 60 cycles/min (26,27) or less (3 to 15 cycles/min) (23,24), we provided cyclic stretch to mesangial cells at 60 cycles/min in the present study. We first examined whether MAPK cascade could be activated by mechanical stretch in mesangial cells. The activities of both p44 and p42 ERKs (ERK1 and ERK2)

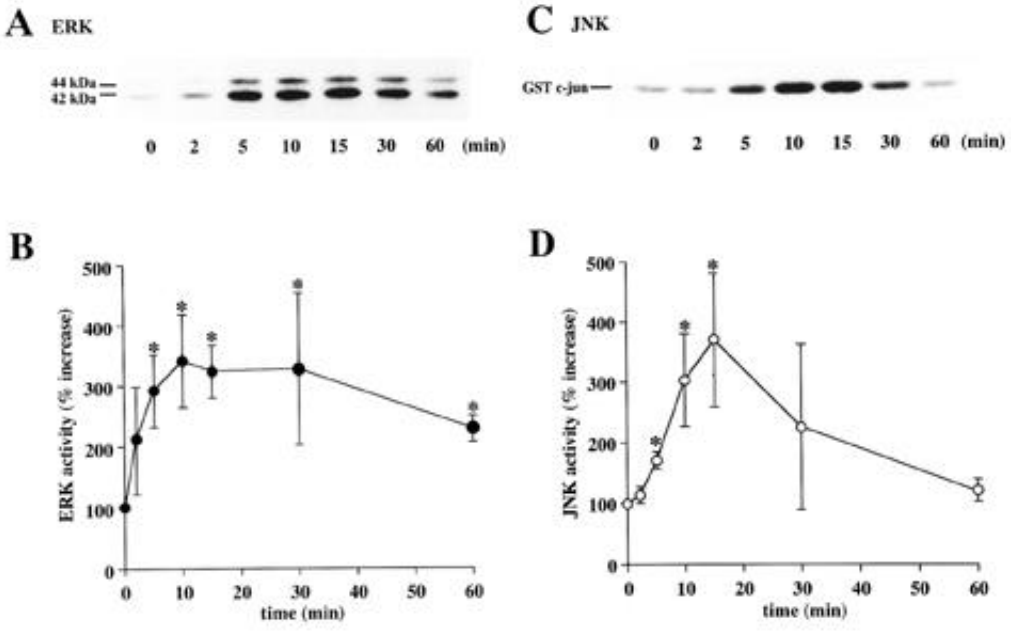

FIG. 1. The activities of ERK and JNK in mesangial cells exposed to mechanical stretch. Quiescent mesangial cells were exposed to cyclic stretch (60 cycles/min; 20% elongation). Cell lysates were obtained at the indicated time points. The activities of ERK were measured by in gel kinase assay using MBP as a substrate, and the activities of JNK were measured by solid-phase kinase assay. *A*: A representative autoradiogram of in gel kinase assay for ERK. *B*: The activities of ERK were quantitated by storage phosphor imaging system. Mean ± SD, $n = 3$, *$P < 0.05$ vs. basal activities. *C*: A representative autoradiogram of solid-phase kinase assay for JNK. *D*: The activities of JNK were quantitated by storage phosphor imaging system. Mean ± SD, $n = 4$, *$P < 0.05$ vs. basal activities.

were enhanced significantly by mechanical stretch from 5 min, and ERKs remained activated at 60 min of stretch (Fig. 1*A* and *B*). The activities of ERKs returned to the basal levels at 120 min of stretch (data not shown). The activity of JNK, one of the members of MAPK family, was also enhanced by mechanical stretch in a time-dependent manner with a peak stimulation at 15 min (Fig. 1*C* and *D*). A retardation in the activation of JNK compared with ERK has been commonly observed in mesangial cells exposed to other stimuli (37,46). As shown in Fig. 2, stretch-induced activation of both ERK and JNK was observed in an intensity-dependent manner.

Mechanical stretch has been reported to activate both PKC (27,28) and PTK (26) in mesangial cells. Since we and others found that ERK could be activated through a PKC- and PTK-dependent mechanism in mesangial cells (36–38), we next examined which pathway could be involved in stretch-induced activation of ERK. The activities of ERK (or MEK) were examined by an immunoblot analysis of active ERK. As shown in Fig. 3, ERK was activated by mechanical stretch in a similar degree as assessed by in gel kinase assay (Fig. 1). The treatment of the cells with herbimycin A, an inhibitor of PTK, before the exposure to mechanical stretch resulted in a significant reduction of stretch-induced phosphorylation of

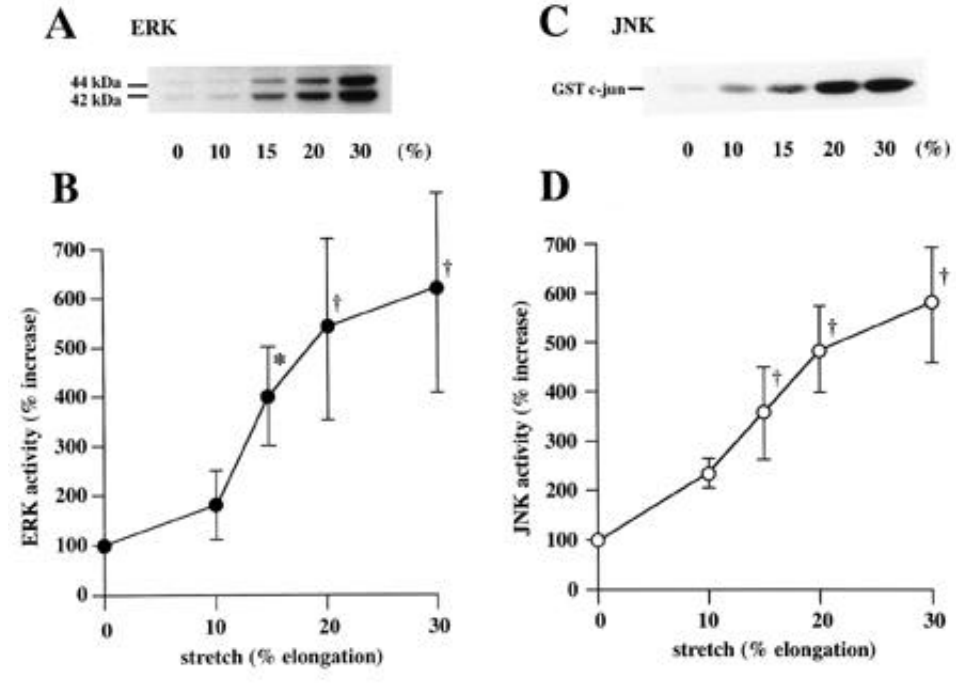

FIG. 2. The intensity-dependent activation of ERK and JNK by mechanical stretch. Quiescent mesangial cells were exposed to cyclic stretch (60 cycles/min) for 15 min at the indicated elongation. *A*: A representative autoradiogram of in gel kinase assay for ERK. *B*: The activities of ERK were quantitated by densitometric analysis. Mean ± SD, $n = 5$, *$P < 0.05$, †$P < 0.01$ vs. nonstretched cells. *C*: A representative autoradiogram of solid-phase kinase assay for JNK. *D*: The activities of JNK were quantitated by densitometric analysis. Mean ± SD, $n = 6$, †$P < 0.01$ vs. nonstretched cells.

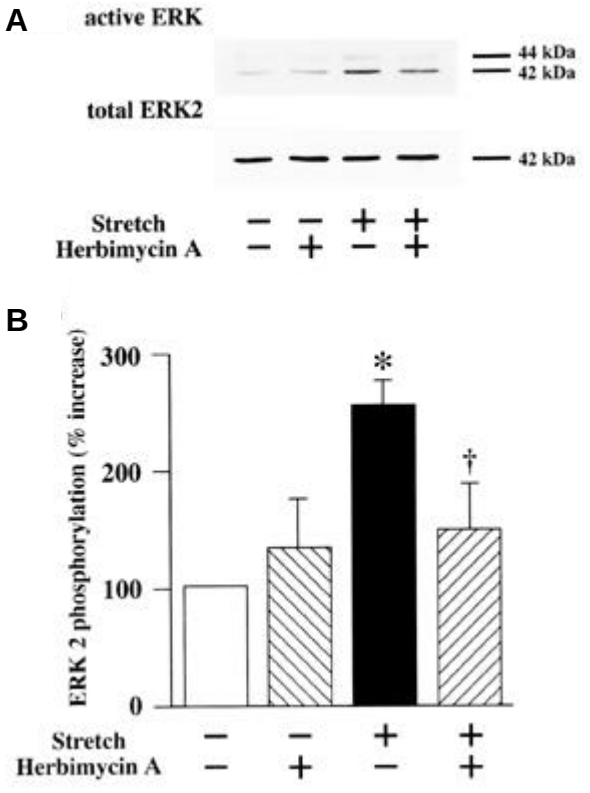

FIG. 3. Effect of PTK inhibitor (herbimycin A) on stretch-induced phosphorylation of ERK. Quiescent mesangial cells were incubated with 1 µmol/l herbimycin A for 16 h and exposed to cyclic stretch for 15 min (60 cycles/min; 20% elongation). The phosphorylation of ERK was detected by immunoblot analysis with anti-active ERK antibody. *A*: A representative result of immunoblot analysis for the effect of herbimycin A on the phosphorylation of ERK. *B*: The phosphorylation of ERK2 was quantitated by densitometric analysis. Mean ± SD, $n = 3$, *$P < 0.01$ vs. control (without stretch), †$P < 0.05$ vs. stretch (without herbimycin A).

ERK (Fig. 3), whereas the treatment with various concentrations of GF109203X or calphostin C, inhibitors of PKC, failed to prevent stretch-induced phosphorylation of ERK (Fig. 4).

Stretch-induced activation of AP-1 in mesangial cells. ERK was found to phosphorylate and activate Elk-1, one of the ternary complex factors, which could enhance the expression of c-*fos* (32), leading to an enhancement of DNA binding activity of AP-1 (33–35). The phosphorylation of c-Jun by JNK could also contribute to the enhancement of DNA binding activity of AP-1 (46,51). Thus, we next examined whether mechanical stretch could enhance DNA-binding activity of AP-1 complex. As shown in Fig. 5*A*, mechanical stretch enhanced DNA-binding activity of AP-1 in a time-dependent manner with a peak stimulation at 60 min. To know the contribution of ERK to stretch-induced enhancement of DNA-binding activity of AP-1, we examined the effect of PD98059, an inhibitor of MEK. As shown in Fig. 5*B*, stretch-induced enhancement of DNA-binding activity of AP-1 was inhibited by treating the cells with PD98059 for 90 min before the exposure to stretch. Because PKC was shown to activate DNA-binding of AP-1 (31), we examined the effect of GF109203X on stretch-induced increase in DNA-binding activities of AP-1. In contrast to PD98059, stretch-induced increase in AP-1 DNA-binding was not affected by treating the cells with 2 µmol/l GF109203X (Fig. 5*C*).

Stretch-induced overproduction of FN and TGF-β1 in mesangial cells. We next examined the expression of FN in mesangial cells exposed to mechanical stretch. The expression of FN mRNA increased from 6 h after stretch (1.94-fold ratio to 36B4) and reached to the maximum at 12 h (2.12-fold). Although the accumulation of FN proteins in the medium gradually increased even in the cells cultured without stretch, the amount of FN produced in the cells exposed to mechanical stretch for 24 h was significantly larger than that in the cells cultured without stretch (data not shown). These results are compatible with the previous reports (23,24,26). To clarify the role of ERK in stretch-induced overproduction of FN, we examined the effect of PD98059, an inhibitor of MEK, on stretch-induced enhancement of the expression of FN. As shown in Fig. 6, mechanical stretch increased the expression of FN and TGF-β1 mRNA, and this stretch-induced increase in the expression of both FN and TGF-β1 mRNA was prevented by treatment with 20 µmol/l PD98059. PD98059 also prevented stretch-induced accumulation of FN in a dose-dependent manner (Fig. 7*A*). As shown in Fig. 7*B* and *C*, PD98059 inhibited stretch-induced activation of ERK in a dose-dependent manner similarly to the results of stretch-induced overproduction of FN (Fig. 7*A*). Although thromboxane-induced increase in FN synthesis was shown to be inhibited by the treatment with 2 µmol/l GF109203X (52,53), stretch-induced production of FN was not inhibited by 2 µmol/l GF109203X (Fig. 7*D*).

DISCUSSION

The present study clearly indicates that MAPKs, ERK and JNK, are activated in glomerular mesangial cells exposed to mechanical stretch, an in vitro model for glomerular hypertension seen in diabetes. The results also suggest that the activation of PTK is responsible for the activation of ERK and the activated ERK may contribute to an overproduction of FN and TGF-β1 in mesangial cells exposed to mechanical stretch.

Glomerular hypertension due to intrarenal hemodynamic changes in diabetes has been reported to contribute to the development and progression of diabetic nephropathy (20,21). An increase in glomerular capillary pressure has been proposed to lead to an enhancement of capillary wall tension, which may cause an expansion of capillary lumen and thus expose mesangial cells to mechanical stretch (22). Recent studies have revealed that mechanical stretch enhances the production of ECM proteins such as FN, laminin, and type IV collagen in mesangial cells (23–27), indicating that mechanical stretch could contribute to the accumulation of ECM proteins in glomerular mesangium. Although the activation of PTK followed by the induction of TGF-β1 (26) or the activation of PKC leading to the activation of S6 kinase (27,28) have been suggested as possible mechanisms underlying stretch-induced enhancement of the production of ECM proteins in mesangial cells, the precise intracellular mechanisms have not been fully elucidated yet.

In the present study, we examined the activities of MAPKs ERK and JNK in mesangial cells exposed to mechanical stretch, because MAPKs were found to play a key role in the expression of various genes, possibly through the enhancement of DNA binding activity of AP-1 (33–35). Both ERK and JNK were activated in mesangial cells by mechanical stretch

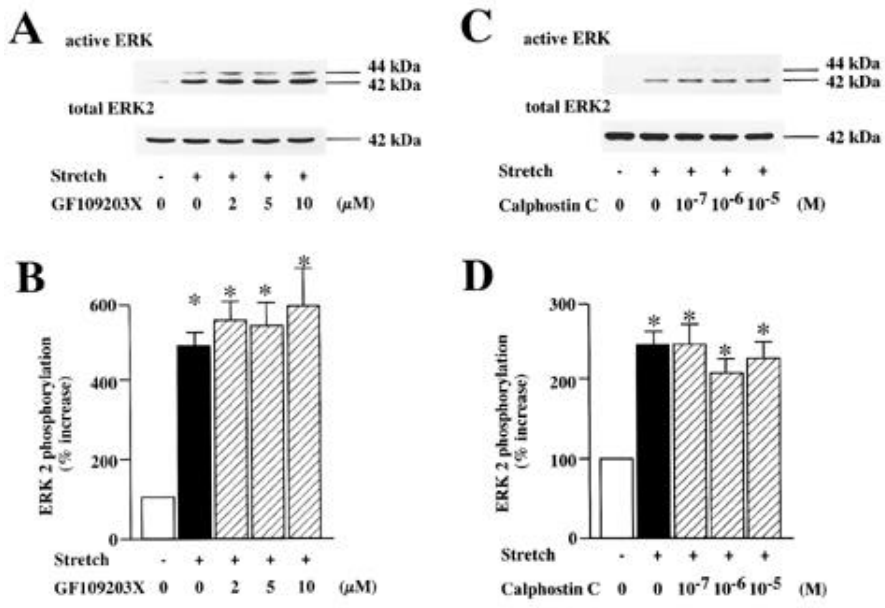

FIG. 4. Effects of PKC inhibitors (GF109203X or calphostin C) on stretch-induced phosphorylation of ERK. Quiescent mesangial cells were incubated with various concentrations of GF109203X for 90 min or calphostin C for 4 h and exposed to cyclic stretch for 15 min (60 cycles/min; 20% elongation). The phosphorylation of ERK was detected by immunoblot analysis with anti–active ERK antibody. *A*: A representative result of immunoblot analysis for the effect of GF109203X on the phosphorylation of ERK. *B*: The phosphorylation of ERK2 was quantitated by densitometric analysis. Mean ± SD, $n = 3$, *$P < 0.01$ vs. control (without stretch). *C*: A representative result of immunoblot analysis for the effect of calphostin C on the phosphorylation of ERK. *D*: The phosphorylation of ERK2 was quantitated by densitometric analysis. Mean ± SD, $n = 3$, *$P < 0.01$ vs. control (without stretch).

in time- and intensity-dependent manners. The activities of ERK remained elevated at 60 min of stretch, while the activities of JNK returned to the basal levels by 60 min. DNA binding activity of AP-1 was enhanced by mechanical stretch. This enhancement was abolished by treating the cells with PD98059, an inhibitor of MEK, indicating that the activation of ERK might be important for the enhancement of DNA binding activity of AP-1 in mesangial cells exposed to mechanical stretch. Although ERK is also activated in mesangial cells cultured under high-glucose conditions (38), the mechanism responsible for the activation of ERK by mechanical stretch seems to be different from that by high glucose. We have shown that the activation of ERK in mesangial cells cultured under high-glucose conditions is dependent on the activation of PKC (38). In the present study, however, the activation of ERK by mechanical stretch was inhibited by herbimycin A, an inhibitor of PTK, but not by GF109203X or calphostin C, inhibitors of PKC. Although both kinases were found to be activated in mesangial cells exposed to mechanical stretch (26–28), the activation of PTK is considered to be responsible for the activation of ERK. A recent report that high pressure, another in vitro model of glomerular hypertension, could activate ERK through PKC-independent mechanisms (54) may support our findings. Since ERK is activated in vivo in glomeruli isolated from diabetic rats (38), the present results and our previous observation (38) suggest that both PKC and PTK may cooperate in vivo to activate ERK. Further study will be necessary to prove this hypothesis.

An importance of the activation of ERK in the functional disturbance of mesangial cells exposed to mechanical stretch is suggested in the present study. The expression of both TGF-β1 and FN was enhanced in mesangial cells exposed to mechanical stretch as reported previously (23,24,26,55). An increase in the expression of both TGF-β1 and FN was prevented by treating the cells with PD98059, an inhibitor of MEK. Therefore, PTK-dependent activation of ERK in mesangial cells exposed to mechanical stretch is considered to be responsible for the stretch-induced overproduction of ECM proteins. In the present experiments, we applied a single intensity of stretch to mesangial cells and

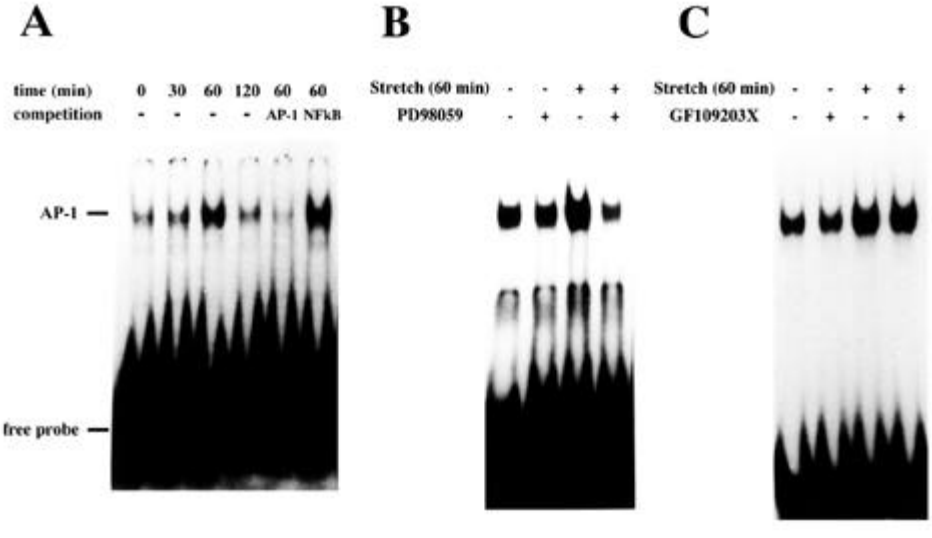

FIG. 5. Mechanical stretch–induced activation of DNA-binding of AP-1. *A*: Quiescent mesangial cells were exposed to cyclic stretch (60 cycles/min; 20% elongation) for the indicated time intervals. Nuclear proteins were obtained at each time point, and the activities of DNA-binding of AP-1 were measured by gel mobility-shift assay. *B*: Mesangial cells were treated with 10 μmol/l PD98059 for 90 min and then exposed to cyclic stretch (60 cycles/min; 20% elongation) for 1 h. *C*: Mesangial cells were treated with 2 μmol/l GF109203X for 90 min and then exposed to cyclic stretch (60 cycles/min; 20% elongation) for 1 h.

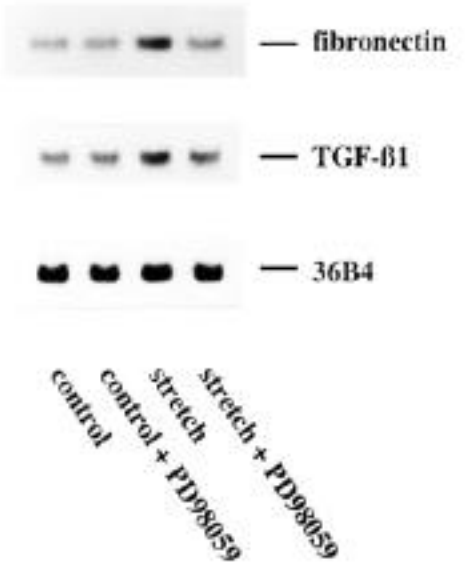

FIG. 6. Effect of MEK inhibitor (PD98059) on stretch-induced expression of FN and TGF-β1 mRNA. Quiescent mesangial cells were treated with 20 μmol/l PD98059 for 90 min and then exposed to cyclic stretch (60 cycles/min; 20% elongation) for 6 h. The mRNA expression of FN, TGF-β1, and 36B4 was measured by Northern blot analysis.

obtained a transient activation of AP-1 DNA binding activity. Since renal arterial blood pressure was found to fluctuate in vivo (56), mesangial cells in vivo might be exposed to stretch transiently or intermittently. Furthermore, recent reports suggest that an intensity of stretch might change in vivo according to the duration of the diabetic milieu (22,57). Thus, stretch-induced activation of AP-1 DNA binding found in the present study might be important in overproduction of FN in vivo. An increase in FN production might be due to the direct induction by AP-1 complex or might result from the induction of TGF-β1. The latter hypothesis is supported by the observation that an increase in AP-1 DNA binding activity could enhance the gene expression of TGF-β1 (58,59). The former possibility is supported by the report that the AP-1 complexes were able to increase the transcriptional activation of FN gene (60). cAMP-responsive elements (CRE) were found in the promoter region of FN gene (61,62), and AP-1 complexes were shown to be able to activate CRE (63). Indeed, phorbol ester and serum have been reported to enhance the gene expression of FN by the activation of CRE (62). These observations suggest that stretch-induced activation of ERK might be able to directly induce the expression of FN gene through the activation of CRE in the FN promoter by enhancing AP-1 DNA binding activity. Further study at the gene level might be necessary to prove this hypothesis.

Mesangial cells are considered to be exposed to stretch stress as well as to high concentrations of glucose in the diabetic milieu. These factors have been reported to enhance the production of ECM proteins independently (5–7,23–27). Since the mechanism responsible for the activation of ERK by mechanical stretch is different from that by high glucose, the additive effects of mechanical stretch and high concentrations of glucose might be present. This possibility is supported by recent findings indicating that both stretch stress

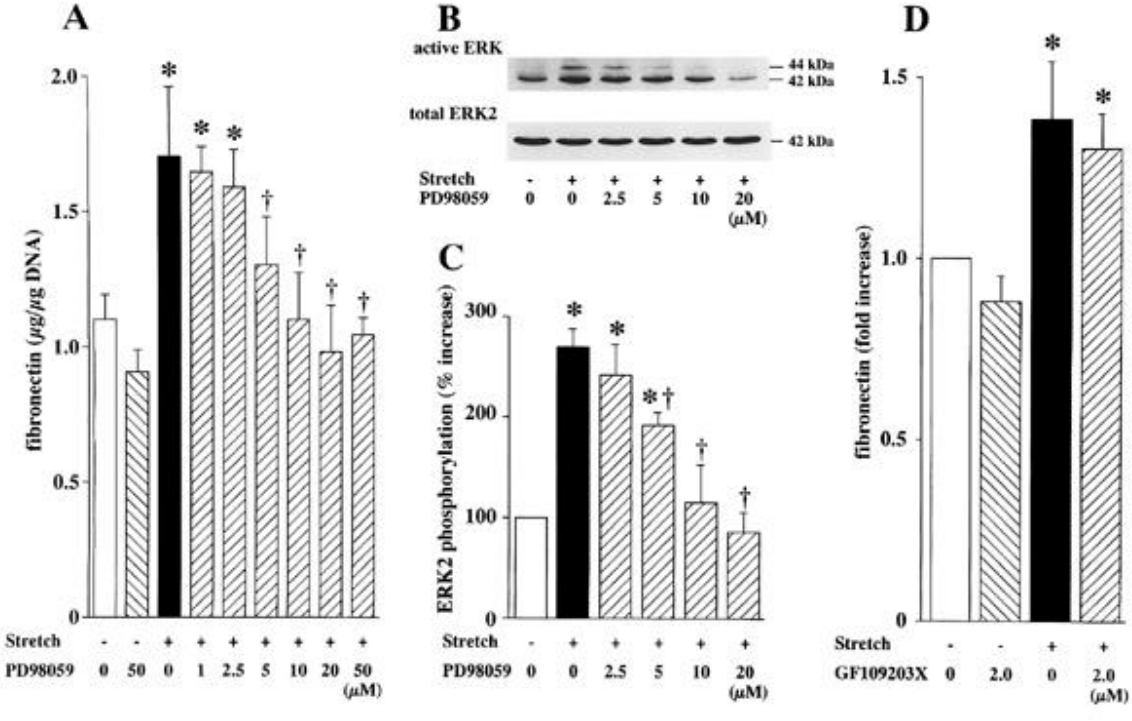

FIG. 7. Effects of MEK inhibitor (PD98059) or PKC inhibitor (GF109203X) on stretch-induced overproduction of FN. The concentrations of FN in cultured medium for 24 h were measured by inhibition ELISA (*A* and *D*), and the phosphorylation of ERK induced by stretch for 15 min was measured by immunoblot analysis (*B* and *C*). Quiescent mesangial cells were treated with various concentrations of PD98059 (*A* to *C*) or 2 μmol/l GF109203X (*D*) for 90 min before exposure to cyclic stretch (60 cycles/min; 20% elongation). *A*: The dose-response effect of PD98059 on stretch-induced accumulation of FN. Mean ± SD, n = 6–9, *P < 0.01 vs. non-stretched cells, †P < 0.01 vs. stretched cells without PD98059. *B*: A representative result of immunoblot analysis for the dose-response effect of PD98059 on the phosphorylation of ERK. *C*: The phosphorylation of ERK2 was quantitated by densitometric analysis. Mean ± SD, n = 4, *P < 0.01 vs. control (without stretch), †P < 0.05 vs. stretch. *D*: The effect of 2 μmol/l GF109203X on stretch-induced accumulation of FN. Mean ± SD, n = 5, *P < 0.05 vs. nonstretched cells.

and high concentrations of glucose act additively in the production of collagens (64), although the precise mechanism of this cooperation has not been clarified. Because mesangial expansion is a major histologic character of diabetic nephropathy, mechanical stretch–induced activation of ERK followed by an increase in the expression of TGF-β1 and FN might contribute to the development and progression of diabetic nephropathy.

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