Applied Pressure Enhances Cell Proliferation through Mitogen-activated Protein Kinase Activation in Mesangial Cells*

Yasunobu Kawata‡, Yoichi Mizukami§¶, Zenzo Fujii‡, Toshihiro Sakumura‡, Ken-ichi Yoshida§, and Masunori Matsuzaki‡

From the Departments of ‡Internal Medicine and §Legal Medicine, Yamaguchi University School of Medicine, 1144 Kogushi, Ube, Yamaguchi 755-8505, Japan

Progressive renal diseases lead to prolonged glomerular hypertension, which induces the proliferation of mesangial cells. This proliferation is thought to be involved in the development of renal injury. Here we investigate mitogen-activated protein kinase (MAPK) activation and cell proliferation in mesangial cells under conditions of high pressure. After pressure-load, the phosphorylation level of MAPK (at Tyr-204) increases rapidly with a peak at 1 min, although the amount of MAPK remains almost constant during pressure-load. To confirm the activation of MAPK, we carried out an immunoprecipitation-kinase assay. MAPK activity during pressure-load shows kinetics similar to that of the tyrosine phosphorylation. In contrast, c-Jun N-terminal kinase 1 (JNK1) phosphorylation falls below basal levels in response to high pressure. Immunocytochemical observations show phosphorylated MAPK in the nucleus at 10 min. The expression of c-Fos, a nuclear transcription factor, is induced by high pressure, and the induction is significantly inhibited by PD98059 (50 μM), an upstream MAPK/extracellular signal-regulated kinase kinase (MEK) inhibitor of MAPK. The expression of the c-Jun that is induced by JNK1 activation remains unchanged during pressure-load. MAPK phosphorylation and cell proliferation by applied pressure are significantly inhibited by genistein, a tyrosine kinase inhibitor in a dose-dependent manner, but not by protein kinase C inhibitors, chelerythrine and GF109203X. Genistein also blocks pressure-induced tyrosine phosphorylation of proteins with molecular masses of 35, 53, and 180 kDa. To clarify the physiological role in MAPK activation under high pressure conditions, we transfected antisense MAPK DNA into mesangial cells. The antisense DNA (2 μM) inhibited MAPK expression by 80% compared with expression in the presence of sense or scrambled DNA, and significantly blocked pressure-induced cell proliferation. Treatment of cells with MEK inhibitor also produced a similar result. MEK inhibitor strongly suppresses DNA synthesis induced by pressure-load. Cyclin D1 expression is significantly increased under high pressure conditions, and the increase is blocked by treatment with MEK inhibitor. These findings show that pressure-load, a novel activator of MAPK, induces the activation of tyrosine kinases, and enhances the proliferation of mesangial cells, probably through cyclin D1 expression.

Mesangial cells in glomeruli are located under a fenestrated capillary endothelium and are exposed to hydrostatic pressure necessary to sustain normal filtration (1–3). Progressive renal diseases, such as diabetic nephropathy, remnant kidney, and hypertensive nephropathy, lead to prolonged glomerular hypertension, which is involved in the mesangial cell proliferation that is considered to be the most important factor mediating glomerular sclerosis (4–11). However, the mechanism of these changes under glomerular hypertension remains largely unknown. Hishikawa *et al.* (12) have reported that, in vascular smooth muscle cells, pressure promotes DNA synthesis and cell growth probably via protein kinase C (PKC),[1] although the detailed mechanism between pressure as an extracellular stimulus and proliferation is unknown.

Various growth factors and mitogenic stimuli are known to induce the activation of mitogen-activated protein kinase (MAPK), a serine/threonine kinase (13, 14). This kinase activity is up-regulated through phosphorylation on tyrosine and threonine residues by MAPK/extracellular signal-regulated kinase kinases (MEKs) (15, 16). MEKs are substrates for Raf-1 (17, 18), which has been reported to be activated either through receptors involved in Ras or a PKC-dependent pathway (19, 20). These MAPK activators cause the translocation of MAPK from the cytosol to the nucleus (21–23), where transcription factors such as Elk-1 (24) and c-Ets (25, 26) are substrates for MAPK. This indicates that MAPK serves as an important regulator of transcriptional activity related to proliferation. Recently, Lavoie *et al.* (27) reported that cyclin D1 expression, which is one of the earliest cell cycle-related events to occur during the G_0/G_1 to S phase transition, is regulated positively by MAPK. Therefore, increasing interest has been paid to the role of MAPK in the cell cycle (28–30). We recently showed that pressure enhances G_1/S progression and promotes the rate of DNA synthesis in mesangial cells (31). However, MAPK activation and its physiological effects in glomerular hypertension are presently unknown. We investigated MAPK activation and cell proliferation in mesangial cells using a pressure-loading apparatus. We show here that applied pressure is a novel activator of MAPK. Furthermore, we demonstrate that MAPK activation plays a role in pressure-induced proliferation, probably via cyclin D1 expression.

EXPERIMENTAL PROCEDURES

Materials—Anti-phospho-MAPK antibody (Tyr-204), anti-phospho-Elk-1 (Ser-383) antibody, Elk-1 fusion protein, and PD98059 (MEK

inhibitor) were bought from New England Biolabs (Beverly, MA). Anti-phospho-JNK1 antibody (Thr-183 and Tyr-185), anti-JNK1 antibody, anti-MAPK antibody, anti-c-Fos antibody, anti-cyclin D1 antibody, peptide substrate of myelin basic protein (APRTPGGRR), and protein A/G-agarose were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-c-Jun antibody and anti-phospho-tyrosine antibody were from Transduction Laboratories (Lexington, KY). Anti-rabbit and anti-mouse immunoglobulin G antibodies coupled to peroxidase were obtained from Promega (Madison, WI). Anti-rabbit immunoglobulin G antibody-linked fluorescein isothiocyanate was from Amersham International plc (Buckinghamshire, UK). The enhanced chemiluminescence reaction assay kit and PKC assay kit were from Pierce. Cell growth was estimated with a cell counting kit (Dojindo Laboratories, Kumamoto, Japan), which is a modified MTT assay kit using WST-1. Genistein, chelerythrine, and GF109203X were from Calbiochem-Novabiochem International (San Diego, CA). All other chemicals were commercially available.

Preparation of Cells—Rat mesangial cells were isolated as described previously (31, 32). Mesangial cells were plated at a density of 5×10^5 cells/dish in 100-mm culture dishes. After incubation in serum-free RPMI 1640 medium for 72 h, the cells were placed for the indicated times under high pressure conditions at 37 °C. High pressure conditions were applied using a pressure-loading apparatus allowing for several levels of air pressure under constant O_2 and CO_2 concentration and temperature as described previously (31). For each time period, cells were collected with a rubber policeman and used for biochemical analyses.

Electrophoresis and Immunoblotting—The collected cells were lysed with lysis buffer (1% Triton X-100, 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM sodium orthovanadate, leupeptin (1 μg/ml), 1 mM phenylmethanesulfonyl fluoride). The cellular extracts and molecular mass standards were electrophoresed in 12.5% (w/v) polyacrylamide gels in the presence of SDS and transferred to polyvinylidene difluoride membranes (0.45 μm, Millipore, Bedford, MA) in the case of phospho-MAPK and phospho-Elk-1, or nitrocellulose membranes for other proteins. The blots were blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.05% (w/v) Tween 20, and incubated with antibody. After the blots were washed, the antigens were visualized by enhanced chemiluminescent detection reagents. The levels of MAPK phosphorylation were determined from the immunoblots by densitometric analysis and were corrected for amounts of MAPK protein.

MAPK Activity and PKC Activity Assay—MAPK activity was determined as described previously (23). Briefly, the cells were lysed in lysis buffer and clarified by centrifugation at 15,000 × g for 10 min at 4 °C. Protein content was normalized, and the lysates were incubated with anti-phospho-MAPK antibody followed by protein A/G-agarose. The complexes were washed twice in lysis buffer and twice in kinase buffer (25 mM Tris (pH 7.5), 5 mM β-glycerol phosphate, 2 mM dithiothreitol, 0.1 mM sodium orthovanadate, 10 mM MgCl₂). Kinase reactions were carried out by resuspending the complexes in 50 μl of kinase buffer containing 100 μM ATP and 1 μg of Elk-1, and incubating for 30 min at 30 °C. The reaction products were electrophoresed in SDS-polyacrylamide (12.5%) gels, transferred to polyvinylidene difluoride membranes, and probed with anti-phospho-Elk-1 antibody. As another method, after cell lysates were incubated with anti-MAPK antibody, MAPK activity was measured by incubating cell extracts for 10 min at 30 °C with 1 mM peptide substrate containing the sequence of myelin basic protein phosphorylated by MAPK (APRTPGGRR) in buffer (25 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 1 mM dithiothreitol, 40 μM ATP, 2 μCi of [γ-³²P]ATP, 2 mM protein kinase inhibitor peptide, 0.5 mM EGTA). The reaction was stopped by the addition of 0.6% HCl, 1 mM ATP, and 1% bovine serum albumin, and the mixture was centrifuged at 3000 × g for 5 min. The supernatant was spotted onto 1.0 × 1.0-cm squares of P81 paper (Whatman), which was washed five times for 10 min each time with 0.5% phosphoric acid, rinsed once with ethanol, dried, and counted by the Cerenkov technique (33). PKC activity was measured with a colorimetric PKC assay kit (Pierce) according to the manufacturer's instructions.

Immunofluorescent Staining of Phospho-MAPK—Mesangial cells were seeded in a Chamber Slide (Lab-Tek, Nunc Inc., Naperville, IL) at a density of 3×10^4 cells/well. The cells were subjected to 70 mm Hg high pressure for 10 min and then fixed with 4% paraformaldehyde in phosphate- buffered saline (PBS) for 15 min at 4 °C. Following fixation, the cells were permeabilized with 0.2% Triton X-100 in PBS for 15 min and blocked with 10% fetal calf serum (FCS) in PBS for 30 min. The cells were then incubated for 1 h at room temperature with antibody against phospho-MAPK at 1:100 dilution in PBS containing 0.1% sheep serum albumin, washed with PBS, and incubated for an additional hour at room temperature with fluorescein isothiocyanate-conjugated anti-
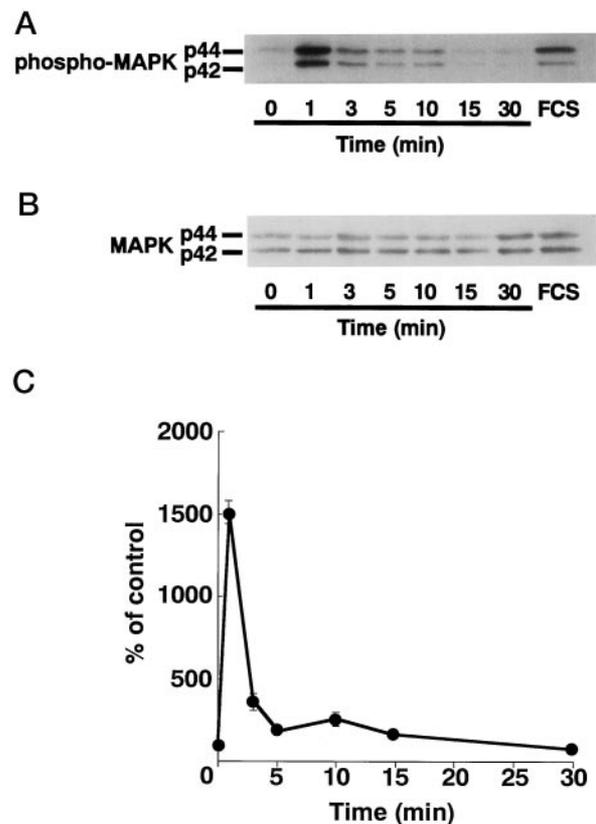

FIG. 1. **Phosphorylation of MAPK in rat mesangial cells under high pressure conditions.** Cell extracts (40 μg of protein) were prepared from mesangial cells exposed to 70 mm Hg high pressure for the indicated times and subjected to immunoblotting with anti-phospho-MAPK antibody (*A*) or anti-MAPK antibody (*B*). A representative immunoblot from three independent experiments is shown. The levels of MAPK phosphorylation were determined from the immunoblots by densitometric analysis (mean ± S.E., *n* = 3) (*C*) as described under "Experimental Procedures."

rabbit immunoglobulin G antibody at 1:100 dilution in PBS. The cells were viewed with a fluorescence microscope (Axioplan 2, Carl Zeiss Co., Heidelberg, Germany).

Transfection of Antisense MAPK into Mesangial Cells—Oligonucleotide transfection was determined as described previously (34). Briefly, the phosphorothioate oligonucleotide with the sequence 5'-GCC GCC GCC GCC GCC AT-3' was synthesized as an antisense DNA. Control phosphorothioate oligonucleotides were synthesized with the following sequences: 5'-ATG GCG GCG GCG GCG GC-3' (sense) and 5'-CGC GCG CTC GCG CAC CC-3' (scrambled). The cells (typically 80% confluent in 24-well dishes) were washed three times with PBS. Appropriate dilutions of oligonucleotides in 200 μl of serum-free RPMI 1640 including liposomes (Tfx-50, Promega Co.) were preincubated at room temperature for 15 min. The cells were incubated for 2 h at 37 °C in the presence of 5% CO₂. At the end of the incubation period, 1 ml of medium containing 10% FCS was added. After incubating for 48 h, the cells were reseeded in 96-well dishes, and incubated with serum-free medium for 24 h after washing with 500 μl of PBS. After a further 24 h the medium was replaced with medium containing 0.5% FCS. After incubation under high pressure conditions, cell number was estimated by WST-1 assay. The densitometric measurements were done with plate analyzer (ETY3A, Toyo Sokki Co., Kanagawa, Japan).

DNA Synthesis—DNA synthesis was estimated with an immunocytochemical assay kit using monoclonal anti-bromodeoxyuridine (BrdUrd) antibody to detect BrdUrd incorporation into cellular DNA (RPN 20, Amersham International). Briefly, growing cells were seeded in chamber slides at a density of 3×10^4/well (0.81 cm²/well). The cells were incubated with serum-free medium for 24 h and then incubated with 0.5% FCS, RPMI 1640 medium under high pressure conditions. After 24 or 48 h, BrdUrd was added to each sample for 30 min, and the cells were then fixed with 4% paraformaldehyde after washing with PBS. After incubation with anti-BrdUrd antibody for 60 min and horseradish peroxidase-conjugated anti-mouse immunoglobulin G antibody
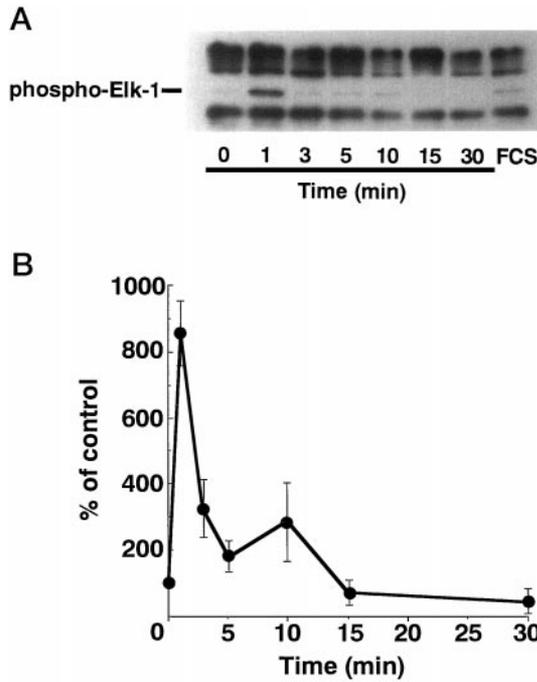
A

phospho-Elk-1 —

0 1 3 5 10 15 30 FCS
Time (min)

B

FIG. 2. **MAPK activity in rat mesangial cells under high pressure conditions.** Cell extracts (600 μg of protein) were prepared from mesangial cells exposed to 70 mm Hg high pressure for the indicated times and subjected to immunoprecipitation with anti-phospho-MAPK antibody followed by protein A/G-agarose. The complexes were incubated with 100 μM ATP and 1.0 μg of Elk-1, and subjected to immunoblotting with anti-phospho-Elk-1 antibody (*A*). A representative immunoblot from three independent experiments is shown. The MAPK activities were determined from the immunoblots by densitometric analysis (mean ± S.E., $n = 3$) (*B*).

for 30 min, BrdUrd incorporation was visualized with 3,3′-diaminobenzidine tetrahydrochloride as a substrate according to the manufacturer's instructions. BrdUrd-positive cells and total cells (200–300 cells) in 10 fields that were selected randomly in each sample were counted, and the proportion of positive cells to total cells was calculated.

RESULTS

MAPK Activation by High Pressure—MAPK is activated through the phosphorylation of Thr-202 and Tyr-204 by MEK (13, 35–37). We initially examined the phosphorylation of MAPK in mesangial cells under high pressure conditions by immunoblotting with an antibody that recognizes phosphorylation at Tyr-204 in MAPK. In pressure-treated cells, their level of MAPK phosphorylation was significantly increased at 1 min after pressure-load as compared with untreated cells (Fig. 1, *A* and *C*). The amount of MAPK remained almost constant throughout pressure-load (Fig. 1*B*). To confirm MAPK activation by high pressure, we examined MAPK activity by an immunoprecipitation kinase assay using Elk-1 as a substrate. MAPK activity rose rapidly after pressure-load with almost the same kinetics as MAPK phosphorylation (Fig. 2, *A* and *B*). To further confirm MAPK activation by high pressure, we carried out another kinase assay using myelin basic protein as described under "Experimental Procedures." MAPK activity with MAPK immunoprecipitates and myelin basic protein peptide, significantly increased (6-fold) with similar kinetics to MAPK phosphorylation and MAPK activity using Elk-1 as a substrate (data not shown). When mesangial cells were exposed to high pressure (30, 50, 70, or 90 mm Hg) or to atmospheric pressure (0 mm Hg), the level of MAPK phosphorylation increased in a pressure-dependent manner (Fig. 3, *A* and *C*). The amount of MAPK remained almost constant in each lane (Fig. 3*B*). These findings show that MAPK is activated by tyrosine phosphorylation in mesangial cells under high pressure conditions. It is
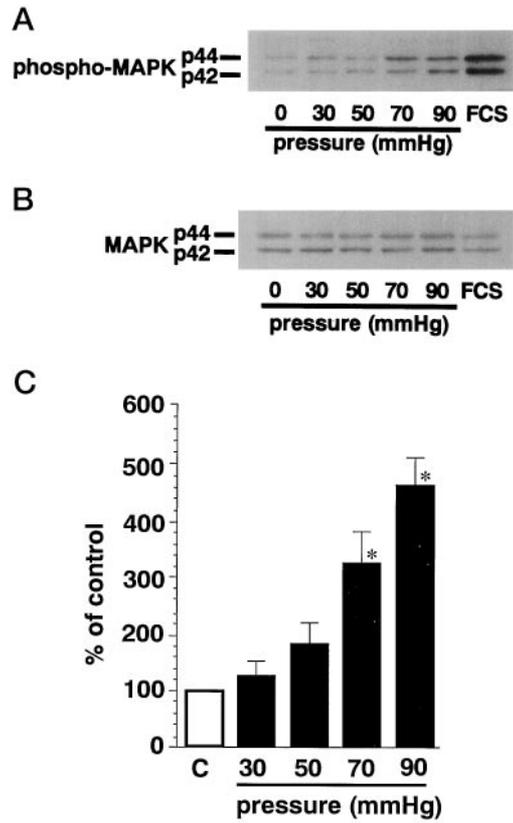
A

phospho-MAPK p44 —
p42 —

0 30 50 70 90 FCS
pressure (mmHg)

B

MAPK p44 —
p42 —

0 30 50 70 90 FCS
pressure (mmHg)

C

FIG. 3. **Effect of pressure-load on MAPK phosphorylation in rat mesangial cells.** Cell extracts (40 μg of protein) were prepared from mesangial cells exposed to the indicated pressures for 3 min and subjected to immunoblotting with anti-phospho-MAPK antibody (*A*) or anti-MAPK antibody (*B*). A representative immunoblot from three independent experiments is shown. The levels of MAPK phosphorylation were determined from the immunoblots by densitometric analysis (mean ± S.E., $n = 3$, *$p < 0.05$ *versus* control) (*C*).
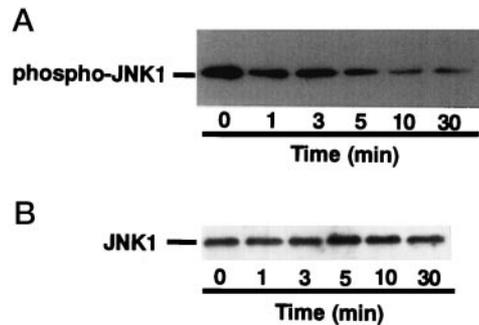
A

phospho-JNK1 —

0 1 3 5 10 30
Time (min)

B

JNK1 —

0 1 3 5 10 30
Time (min)

FIG. 4. **Phosphorylation of JNK1 in rat mesangial cells under high pressure conditions.** Cell extracts (40 μg of protein) were prepared from mesangial cells exposed to 70 mm Hg high pressure for the indicated times, and subjected to immunoblotting with anti-phospho-JNK1 antibody (*A*) or anti-JNK1 antibody (*B*). A representative immunoblot from three independent experiments is shown.

known that JNK1, a member of the MAPK superfamily, is activated by phosphorylation by SEK1 in response to various stresses (38–41). We measured the level of JNK1 phosphorylation necessary for activation by immunoblotting with an anti-phospho-JNK1 antibody. As shown in Fig. 4*A*, JNK1 was already phosphorylated under the control conditions, and the level of phosphorylation was rather lower during pressure-load, although the amount of JNK1 remained almost unchanged during pressure-load (Fig. 4*B*). These results indicate that JNK1 phosphorylation decreases during pressure-load.

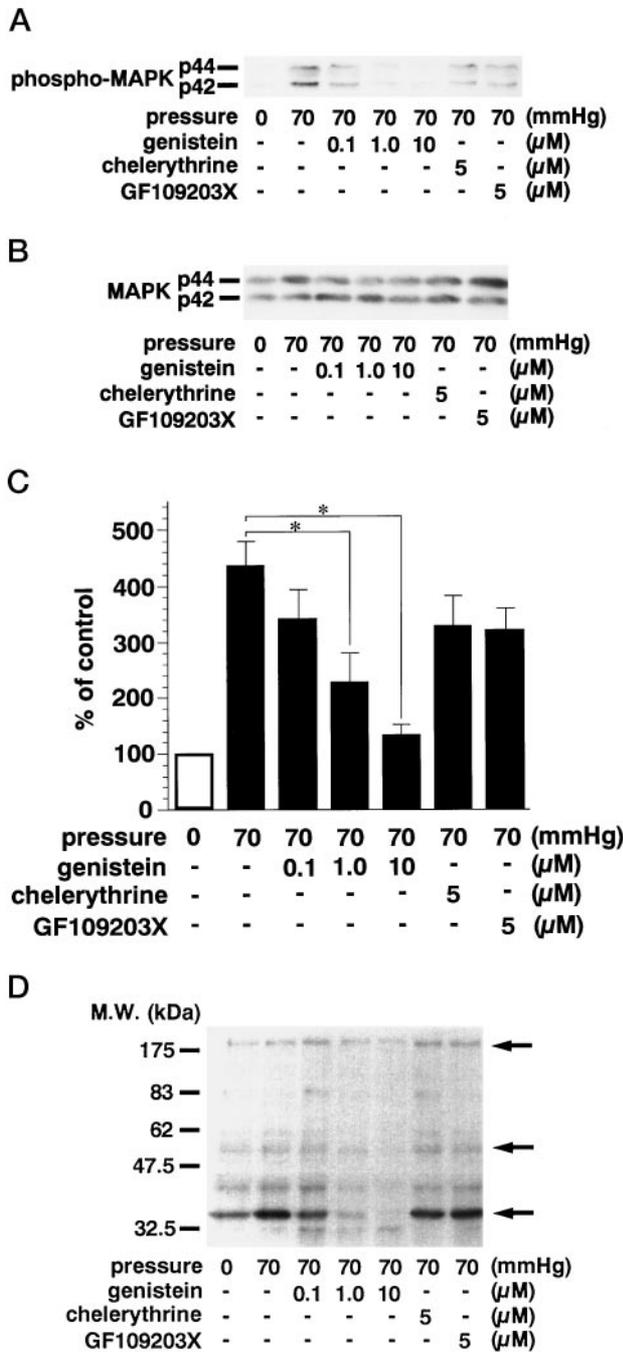To investigate upstream kinases in the pressure-induced

A

B

C

D

FIG. 6. **Subcellular localization of phospho-MAPK in mesangial cells under high pressure conditions.** The mesangial cells were incubated for 0 min (*A* and *B*) or 10 min (*C* and *D*) at 37 °C under the condition of 70 mm Hg high pressure, fixed in 4% paraformaldehyde, and stained with anti-phospho-MAPK antibody as described under "Experimental Procedures." Phase-contrast microscopic photographs were shown in *A* and *C*. A representative photograph from three independent experiments is shown. Final magnification, × 400.

A

B

FIG. 7. **Expression of c-Fos in rat mesangial cells under high pressure conditions.** Cell extracts (40 μg of protein) were prepared from mesangial cells exposed to 70 mm Hg high pressure for the indicated times, and subjected to immunoblotting with anti-c-Fos antibody (*A*). A representative immunoblot from three independent experiments is shown. The amount of c-Fos was determined from the immunoblots by densitometric analysis (mean ± S.E., $n = 3$, *$p < 0.05$ versus control) (*B*).

FIG. 5. **Effect of various inhibitors on pressure-induced MAPK phosphorylation and tyrosine phosphorylation.** Cell extracts (40 μg protein) were prepared from mesangial cells exposed to 70 mm Hg high pressure for 3 min in the presence of each inhibitor and subjected to immunoblotting with anti-phospho-MAPK antibody (*A*), anti-MAPK antibody (*B*), or anti-phopho-tyrosine antibody (*D*). A representative immunoblot from three independent experiments is shown. The levels of MAPK phosphorylation were determined from the immunoblots by densitometric analysis (mean ± S.E., $n = 3$, *$p < 0.05$) (*C*).

MAPK activation, we examined the MAPK phosphorylation in the presence of inhibitors for PKC and tyrosine kinases. Genistein, a tyrosine kinase inhibitor, inhibited pressure-induced MAPK phosphorylation in a dose-dependent manner (Fig. 5, *A* and *C*). However, chelerythrine and GF109203X, PKC inhibitors, did not affect MAPK phosphorylation (Fig. 5, *A* and *C*). The amount of MAPK was almost constant in each lane (Fig. 5*B*). Furthermore, to confirm the effect of genistein, we examined tyrosine phosphorylation with immunoblotting using anti-
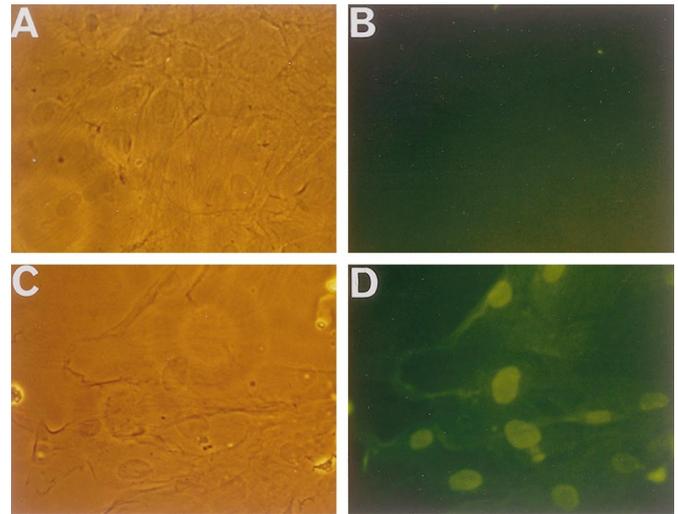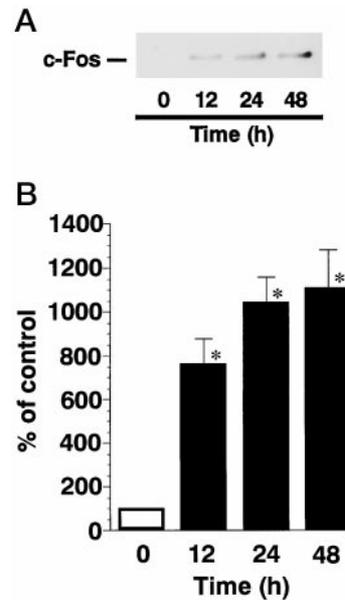
phospho-tyrosine antibody. Applied pressure induces the tyrosine phosphorylation of proteins with molecular masses of 35, 53, and 180 kDa, and the tyrosine phosphorylation was inhibited by genistein (Fig. 5*D*) in a dose-dependent manner (0.1–10 μM). PKC inhibitors had no effects on the pressure-induced tyrosine phosphorylation (Fig. 5*D*). Genistein also inhibited pressure-induced cell proliferation, whereas PKC inhibitors had no effect (data not shown). PKC activity was weakly increased by applied pressure, the activity of which was blocked by PKC inhibitors, but not by 10 μM genistein (data not shown). These findings suggest that tyrosine kinases are involved in
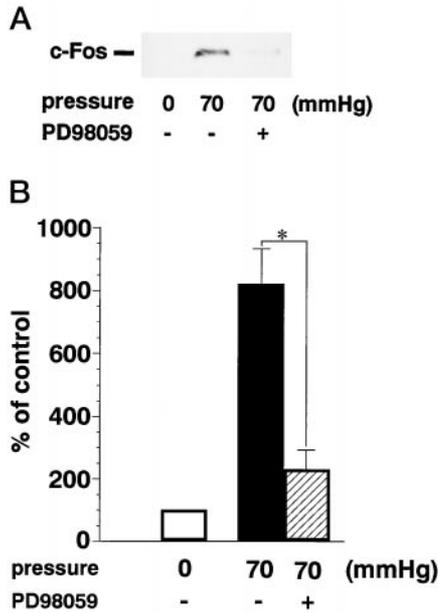
A

c-Fos —

pressure	0	70	70	(mmHg)
PD98059	-	-	+	

B

FIG. 8. **Effect of MEK inhibitor on c-Fos expression in mesangial cells under high pressure conditions.** Cell extracts (40 μg of protein) were prepared from the mesangial cells exposed to 70 mm Hg high pressure for 24 h in the presence or absence of MEK inhibitor (50 μM PD98059) and subjected to immunoblotting with anti-c-Fos antibody (*A*). A representative immunoblot from three independent experiments is shown. The amount of c-Fos was determined from the immunoblots by densitometric analysis (mean $\pm$ S.E., $n = 3$, $*p < 0.05$) (*B*).

c-Jun —

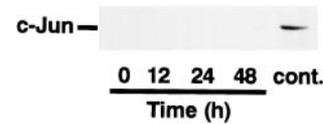|	0	12	24	48	cont.
			Time (h)		

FIG. 9. **Expression of c-Jun in rat mesangial cells under high pressure conditions.** Cell extracts (40 μg of protein) were prepared from mesangial cells exposed to 70 mm Hg high pressure for the indicated times and subjected to immunoblotting with anti-c-Jun antibody. A representative immunoblot from three independent experiments is shown.

the pressure-induced MAPK activation.

MAPK substrates are present in various subcellular fractions (21, 22). To examine the physiological effect of MAPK under high pressure conditions, we observed the subcellular localization of MAPK by immunocytochemical staining using an anti-phospho-MAPK antibody. Immunocytochemical observations showed the phosphorylated MAPK to be present mainly in the cytoplasm 1 min after pressure-load (data not shown), and the nuclear staining was significantly increased after 10 min of pressure-load (Fig. 6, *C* and *D*). The staining was not observed under control conditions (atmospheric pressure) (Fig. 6, *A* and *B*) and was inhibited by preincubating the primary antibody with activated MAPK (data not shown). These findings suggest that MAPK has a physiological effect on the nucleus.

It is known that MAPK in the nucleus can phosphorylate Elk-1 (24), a transcription factor, and that this phosphorylation subsequently leads to an increase in the expression of c-Fos (42). We examined the expression of c-Fos under high pressure conditions by immunoblotting and found it to be significantly increased for 12 h after pressure-load (Fig. 7, *A* and *B*). This increase was inhibited by treatment with 50 μM MEK inhibitor (Fig. 8, *A* and *B*). In addition, antisense DNA against MAPK, as described below, also inhibits pressure-induced c-Fos expression (data not shown). On the other hand, the expression of c-Jun, a nuclear transcription factor that is induced by JNK activation, remained almost unchanged under 70 mm Hg pressure for up to 48 h (Fig. 9). These observations demonstrate that applied pressure induces the expression of c-Fos via the activation of MAPK.

Cell Proliferation through MAPK Activation by High Pressure—We recently showed that high pressure enhances the rates of DNA synthesis and cell proliferation (31). To investigate the role of MAPK in pressure-induced proliferation, we transfected an antisense oligonucleotide against MAPK into mesangial cells, and found MAPK expression to be inhibited in a concentration-dependent manner (0.1–2.0 μM) (Fig. 10, *A* and *B*). Incubation of cells with 2 μM sense or scrambled DNA for MAPK did not significantly affect MAPK expression for up to 4

days. JNK1 expression remained almost unchanged in cells transfected with each MAPK DNA as compared with untreated cells. We examined cell proliferation using the WST-1 assay. Mesangial cells whose MAPK protein was depleted by antisense DNA, completely inhibited pressure-induced proliferation, but sense DNA or scrambled DNA-treated cells enhanced proliferation to the same extent as untreated cells for up to 48 h under high pressure conditions (Fig. 11). Cell treatment with MEK inhibitor produced similar results in terms of cell numbers (data not shown). DNA synthesis in mesangial cells was enhanced under 70 mm Hg high pressure condition as described previously (31). This enhancement was strongly blocked by 50 μM MEK inhibitor (Fig. 12). These findings show that MAPK activation participates in pressure-induced proliferation. In addition, the applied pressure for 30 min weakly induced the cell proliferation at 24 h under the condition of atmospheric pressure, but not at 48 h (data not shown), which suggests that transient pressure can proliferate mesangial cells.

It was recently reported that cyclin D1 expression, which is required to pass the G_1 restriction point, is positively regulated by MAPK (27). We examined cyclin D1 expression under high pressure conditions by immunoblotting using an anti-cyclin D1 antibody. The expression of cyclin D1 was induced after 12 h of pressure-load (Fig. 13, *A* and *B*), and the expression was significantly suppressed to basal levels by MEK inhibitor (Fig. 14, *A* and *B*). The results suggest that cyclin D1, induced by MAPK activation, may be involved in cell proliferation under high pressure conditions.

DISCUSSION

We have investigated MAPK activation in mesangial cells exposed to high pressure. During the study we demonstrated that a few minutes of pressure-load induces the activation and nuclear translocation of MAPK, which leads to an increase in the expression of c-Fos during the pressure-load. MAPK activation under high pressure conditions increases cyclin D1 expression and DNA synthesis, and finally enhances cell cycle progression. In addition, pressure-induced MAPK activation and cell proliferation might be involved in tyrosine kinases.

MAPK is strongly activated by growth factors and growth-promoting hormones (13, 43–47), in contrast to JNK, which is preferentially activated by environmental stresses and pro-inflammatory cytokines (14, 38, 48, 49). In this study, applied pressure, which is a physical force generated by hydrostatic pressure, promoted the activation of MAPK but not JNK1. MAPK has been reported to phosphorylate Elk-1, a nuclear transcription factor (50, 51). Elk-1 binds to a serum response element within the c-*fos* promoter region together with a serum response factor, inducing c-Fos expression (42, 52, 53). High pressure induces c-Fos expression, and the induction is inhibited by both MAPK antisense oligonucleotide and MEK inhibitor. These observations demonstrate that MAPK activation is involved in the expression of c-Fos under high pressure conditions. A transcription factor, c-Ets, is also a substrate for MAPK (54, 55). The promoter region of cyclin D1 has an Ets-like binding domain that regulates cyclin D1 expression (25,

A

MAPK p44—
p42—

0 0.1 0.5 2.0   0.1 0.5 2.0 (*µ*M)
    sense DNA   antisense DNA

FIG. 10. **Effect of MAPK antisense DNA on MAPK expression in rat mesangial cells under high pressure conditions.** Cell extracts (20 µg of protein) were prepared from mesangial cells transfected with antisense DNA or sense DNA and subjected to immunoblotting with anti-MAPK antibody (*A*). A representative immunoblot from two independent experiments is shown. The amount of MAPK was determined from the immunoblots by densitometric analysis (*B*).
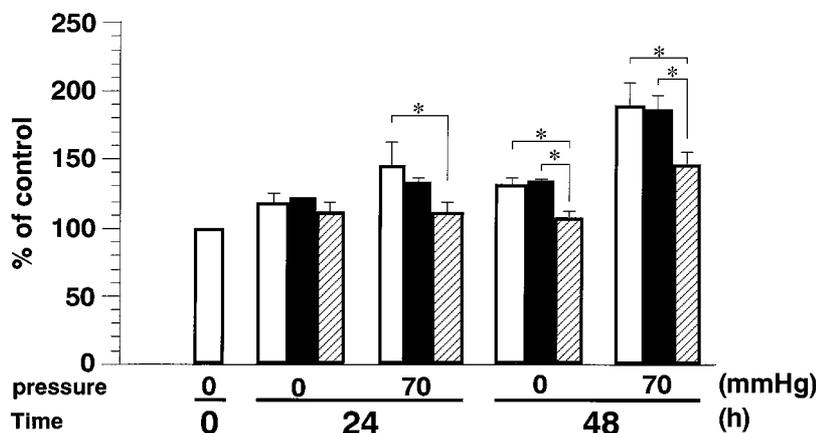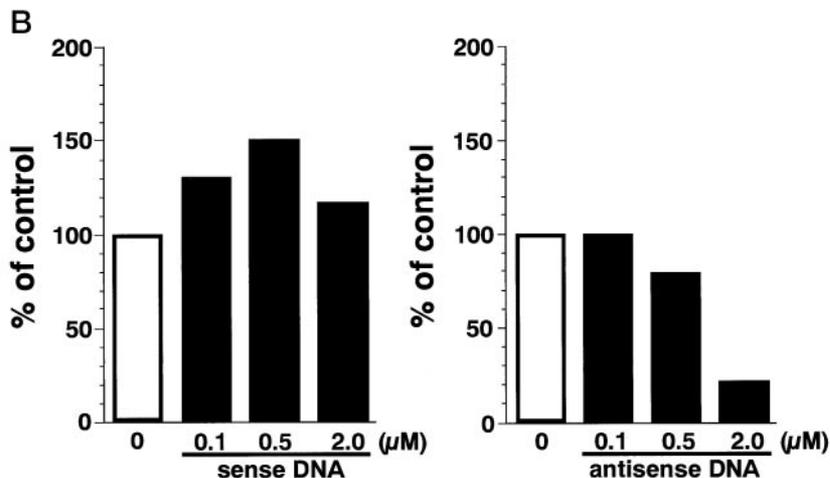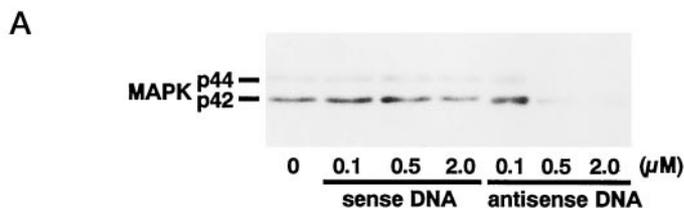
B

FIG. 11. **Effect of MAPK antisense DNA on the proliferation of mesangial cells under high pressure conditions.** Mesangial cells were pretreated with 2 µM of scrambled DNA (*open column*), sense DNA (*closed column*), or antisense DNA (*hatched column*) for 2 h in RPMI 1640 containing liposomes (Tfx-50). Cell numbers under the conditions of 0 mm Hg (atmospheric pressure) or 70 mm Hg (high pressure) for the indicated times were determined by WST-1 assay (mean ± S.E., $n = 4$, *$p < 0.05$) as described under "Experimental Procedures."

54). Recently, Lavoie *et al.* (27) reported that MAPK plays a positive regulatory role in cyclin D1 expression. Consistent with their report, our present data show that pressure-load activates MAPK, which increases cyclin D1 expression and an enhancement of DNA synthesis and cell growth. Cyclin D1 plays an important role in the entry of cells into S phase and cell cycle progression (56–59). Therefore, cyclin D1 expression may participate in pressure-induced proliferation since pressure-load contributes to cell cycling by enhancing G_1/S progression and promoting the rate of DNA synthesis in mesangial cells as described previously (31).

The mechanism of MAPK activation under high pressure conditions is poorly elucidated. It does not appear that mesangial cells secrete growth factors that would activate MAPK during pressure-load for the following two reasons. First, we added a supernatant from pressure-treated cells to untreated cells and observed no cell proliferation under the experimental conditions employed (data not shown). This result is consistent with studies on smooth muscle cells as described previously (12). Second, MAPK is rapidly activated, reaching a peak at 1 min after pressure-load; there seems to be no time delay due to the secretion of growth factors after pressure-load. Hishikawa *et al.* (12) have reported that pressure promotes the activation

of PKC with Ca^{2+} mobilization in cultured smooth muscle cells. It is well known that various stresses activate distinct PKC isoforms (60–63). In mesangial cells as well as smooth muscle cells, it is possible that pressure-load activates PKC isoforms. In our study, PKC activity was weakly increased by applied pressure. However, pressure-induced MAPK phosphorylation was inhibited by tyrosine kinase inhibitor, but not by PKC inhibitors. In addition, applied pressure induces tyrosine phosphorylation of proteins with molecular masses of 180, 53, and 35 kDa, and tyrosine phosphorylation was inhibited by genistein. Thus, it is strongly suggested that tyrosine kinases are involved in the pressure-induced MAPK activation.

MAPK activation in mesangial cells is thought to play an important role in the development of renal injury. Pressure-load itself activates MAPK, which contributes to the mesangial cell proliferation that is a central feature of numerous glomerular diseases (8–11). In addition, Bokemeyer *et al.* (64) have recently reported that MAPK is significantly activated by proliferative glomerulonephritis in response to immune injury. The proliferation induced by renal diseases is an important aspect of the pathogenic process of glomerular sclerosis (64). In these diseases, this is often accompanied not only by the proliferation of mesangial cells but also by expansion of the extra-
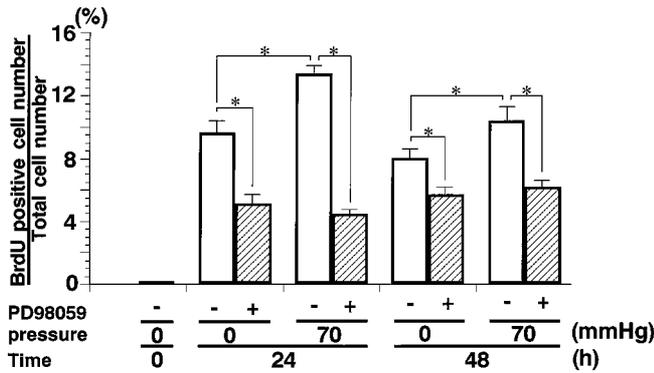
FIG. 12. **Effect of MEK inhibitor on DNA synthesis in mesangial cells under high pressure conditions.** Mesangial cells were incubated in the presence or absence of MEK inhibitor (50 μM; PD98059) under the conditions of 0 mm Hg (atmospheric pressure) or 70 mm Hg (high pressure) for the indicated times. DNA synthesis was determined by immunocytochemical assay using anti-BrdUrd (*BrdU*) antibody to detect BrdUrd incorporation into cellular DNA as described under "Experimental Procedures." Ten fields were examined in a sample, BrdUrd-positive cells in total cells (200–300 cells) were counted in each field, and the proportion of BrdUrd-positive cells to total cells was calculated (mean $\pm$ S.E., $n = 4$, *$p < 0.05$).
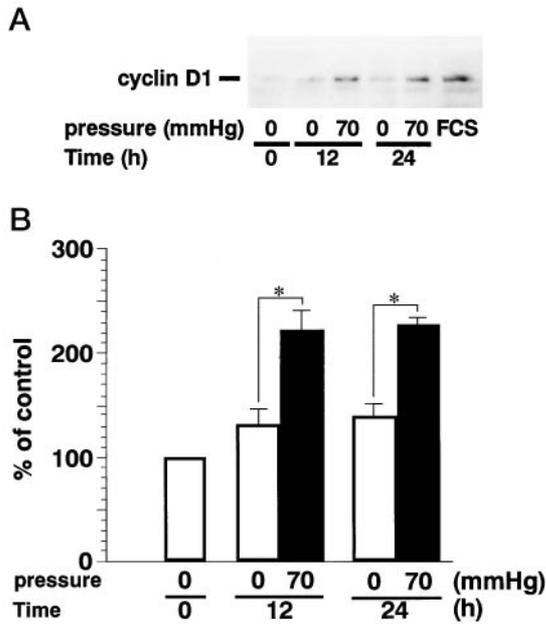

FIG. 13. **Expression of cyclin D1 in rat mesangial cells under high pressure conditions.** Cell extracts (40 μg of protein) were prepared from mesangial cells exposed to 0 mm Hg (atmospheric pressure) or 70 mm Hg (high pressure) for the indicated times and subjected to immunoblotting with anti-cyclin D1 antibody (*A*). A representative immunoblot from three independent experiments is shown. The amount of cyclin D1 was determined from the immunoblots by densitometric analysis (mean $\pm$ S.E., $n = 3$, *$p < 0.05$) (*B*).

cellular matrix (65–68). Transforming growth factor-β (TGF-β) is reported to be a factor in regulating the extracellular matrix and inducing glomerular sclerosis (67–69). It is known that the promoter region of TGF-β contains an AP1 element (70–73) and that c-Fos expression regulates TGF-β expression. In our study, we found that pressure-load significantly increases the expression of TGF-β in mesangial cells for 24 h.[2] Pressure-load may increase TGF-β expression through c-Fos induced by MAPK activation. It is possible that pressure-load contributes to the development of renal injury by both mesangial prolifera-
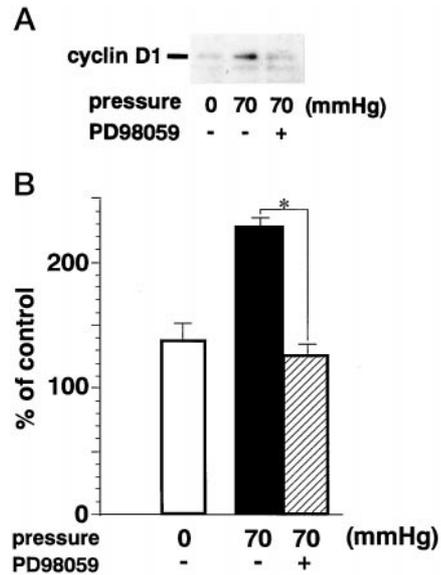
---

[2] Y. Kawata, Y. Mizukami, Z. Fujii, K. Yoshida, and M. Matsuzaki, unpublished data.

FIG. 14. **Effect of MEK inhibitor on cyclin D1 expression in mesangial cells under high pressure conditions.** Cell extracts (40 μg of protein) were prepared from the mesangial cells exposed to 0 mm Hg (atmospheric pressure) or 70 mm Hg (high pressure) for 24 h in the presence or absence of MEK inhibitor (50 μM; PD98059), and subjected to immunoblotting with anti-cyclin D1 antibody (*A*). A representative immunoblot from three independent experiments is shown. The amount of cyclin D1 was determined from the immunoblots by densitometric analysis (mean $\pm$ S.E., $n = 3$, *$p < 0.05$) (*B*).

tion and matrix expansion through MAPK activation. This MAPK activation of mesangial cells in response to pressure-load might be an underlying mechanism in the development of renal injury with glomerular hypertension. The present study provides new insights into the role of MAPK in glomerular hypertension.

REFERENCES

1. Kriz, W., Elger, M., Mundel, P., and Lemley, K. V. (1995) *J. Am. Soc. Nephrol.* **5,** 1731–1739
2. Kriz, W., Elger, M., Lemley, K., and Sakai, T. (1990) *Kidney Int.* **38,** Suppl. 30, S2–S9
3. Cortes, P., Riser, B. L., Zhao, X., and Narins, R. G. (1994) *Kidney Int.* **45,** S11–S16
4. Ofstad, J., Horvei, G., Kvam, F. I., Morkrid, L., Sekse, I., Svarstad E., Iversen, B. M. (1992) *Kidney Int.* **41,** S8–S14
5. Hostetter, T. H., Olson, J. L., Rennke, H. G., Venkatachalam, M. A., and Brenner, B. M. (1981) *Am. J. Physiol.* **241,** F85–F93
6. Hostetter, T. H., Troy, J. L., and Brenner, B. M. (1981) *Kidney Int.* **19,** 410–415
7. Dworkin, L. D. and Feiner, H. D. (1986) *J. Clin. Invest.* **77,** 797–809
8. Dworkin, L. D., Hostetter, T. H., Rennke, H. G., and Brenner, B. M. (1984) *J. Clin. Invest.* **73,** 1448–1461
9. Anderson, S., Meyer, T. W., Rennke, H. G., and Brenner, B. M. (1985) *J. Clin. Invest.* **76,** 612–619
10. Brenner, B. M., Meyer, T. W., and Hostetter, T. H. (1982) *N. Engl. J. Med.* **307,** 652–659
11. Zatz, R., Dunn, B. R., Meyer, T. W., Anderson, S., Rennke, H. G., and Brenner, B. M. (1986) *J. Clin. Invest.* **77,** 1925–1930
12. Hishikawa, K., Nakaki, T., Marumo, T., Hayashi, M., Suzuki, H., Kato, R., and Saruta, T. (1994) *J. Clin. Invest* **93,** 1975–1980
13. Nishida, E. and Gotoh, Y. (1993) *Trends Biochem. Sci.* **18,** 128–131
14. Karin, M. (1992) *FASEB J.* **6,** 2581–2590
15. Canagarajah, B. J., Khokhlatchev, A., Cobb, M. H., and Goldsmith, E. J. (1997) *Cell* **90,** 859–869
16. Zanke, B. W., Rubie, E. A., Winnett, E., Chan, J., Randall, S., Parsons, M., Boudreau, K., McInnis, M., Yan, M., Templeton, D. J., and Woodgett, J. R. (1996) *J. Biol. Chem.* **271,** 29876–29881
17. Kyriakis, J. M., App, H., Zhang, X.-F., Banerjee, P., Brautigan, D. L., Rapp, U. R., and Avruch, J. (1992) *Nature* **358,** 417–421
18. Lange-Carter, C. A., Pleiman, C. M., Gardner, A. M., Blumer, K. J., and Johnson, G. L. (1993) *Science* **260,** 315–319
19. Marshall, C. J. (1994) *Curr. Opin. Genet. Dev.* **4,** 82–89
20. Kolch, W., Heidecker, G., Kochs, G., Hummel, R., Vahidi, H., Mischak, H., Finkenzeller, G., Marme, D., and Rapp, U. R. (1993) *Nature* **364,** 249–252
21. Lenormand, P., Sardet, C., Pages, G., L'Allemain, G., Brunet, A., and Pouyssegur, J. (1993) *J. Cell Biol.* **122,** 1079–1088
22. Gonzalez, F. A., Seth, A., Raden, D. L., Bowman, D. S., Fay, F. S., and Davis, R. J. (1993) *J. Cell Biol.* **122,** 1089–1101
23. Mizukami, Y. and Yoshida, K. (1997) *Biochem. J.* **323,** 785–790
24. Janknecht, R., Ernst, W. H., Pingoud, V., and Nordheim, A. (1993) *EMBO J.* **12,** 5097–5104
25. Albanese, C., Johnson, J., Watanabe, G., Eklund, N., Vu, D., Arnold, A., and

Pestell, R. G. (1995) *J. Biol. Chem.* **270,** 23589–23597

26. McCarthy, S. A., Chen, D., Yang, B. S., Garcia Ramirez, J. J., Cherwinski, H., Chen, X. R., Klagsbrun, M., Hauser, C. A., Ostrowski, M. C., and McMahon, M. J. (1997) *Mol. Cell. Biol.* **17,** 2401–2412
27. Lavoie, J. N., L'Allemain, G., Brunet, A., Muller, R., and Pouyssegur, J. (1996) *J. Biol. Chem.* **271,** 20608–20616
28. Takeda, T., Toda, T., Kominami, K., Kohnosu, A., Yanagida, M., and Jones, N. (1995) *EMBO J.* **14,** 6193–6208
29. Wilkinson, M. G., Samuels, M., Takeda, T., Toone, W. M., Shieh, J. C., Toda, T., Millar, J. B., and Jones N. (1996) *Genes Dev.* **10,** 2289–2301
30. Pombo, C. M., Bonventre J. V., Molnar, A., Kyriakis, J., and Force, T. (1996) *EMBO J.* **15,** 4537–4546
31. Kawata, Y., Fujii, Z., Sakumura, T., Kitano, M., Suzuki, N., and Matsuzaki, M. (1998) *Biochim. Biophys. Acta* **1401,** 195–202
32. Harper, P. A., Robinson, J. M., Hoover, R. L., Wright, T. C., and Karnovsky, M. J. (1984) *Kidney Int.* **26,** 875–880
33. Saunier, B., Tournier, C., Jacquemin, C., and Pierre, M. (1995) *J. Biol. Chem.* **270,** 3693–3697
34. Sale, E. M., Atkinson, P. G., and Sale, G. J. (1995) *EMBO J.* **14,** 674–684
35. Crews, C. M., Alessandrini, A., and Erikson, R. L. (1992) *Science* **258,** 478–480
36. Pelech, S. L. and Sanghera, J. S. (1992) *Trends Biochem. Sci.* **17,** 233–238
37. Sturgill, T. W., Ray, L. B., Erikson, E., and Maller, J. L. (1988) *Nature* **334,** 715–718
38. Kyriakis, J. M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E. A., Ahmad, M. F., Avruch, J., and Woodgett, J. R. (1994) *Nature* **369,** 156–160
39. Mizukami, Y., Yoshioka, K., Morimoto, S., and Yoshida, K. (1997) *J. Biol. Chem.* **272,** 16657–16662
40. Devary, Y., Gottlieb, R. A., Smeal, T., and Karin, M. (1992) *Cell* **71,** 1081–1091
41. Yan, M., Dai, T., Deak, J. C., Kyriakis, J. M., Zon, L. I., Woodgett, J. R., and Templeton, D. J. (1994) *Nature* **372,** 798–800
42. Gille, H., Sharrocks, A. D., and Shaw, P. E. (1992) *Nature* **358,** 414–417
43. Bianchini, L., L'Allemain, G., and Pouyssegur, J. (1997) *J. Biol. Chem.* **272,** 271–279
44. Brunet, A. and Pouyssegur, J. (1996) *Science* **272,** 1652–1655
45. Lange-Carter, C. A. and Johnson, G. L. (1994) *Science* **265,** 1458–1461
46. Selva, E., Raden, D. L., and Davis, R. J. (1993) *J. Biol. Chem.* **268,** 2250–2254
47. Blenis, J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 5889–5892
48. Derijard, B., Hibi, M., Wu, I. H., Barrett, T., Su, B., Deng, T., Karin, M., and Davis, R. J. (1994) *Cell* **76,** 1025–1037
49. Force, T., Pombo, C. M., Avruch, J. A., Bonventre, J. V., and Kyriakis, J. M. (1996) *Circ. Res.* **78,** 947–953
50. Marais, R., Wynne, J., and Treisman, R. (1993) *Cell* **73,** 381–393
51. Gille, H., Kortenjann, M., Thomae, O., Moomaw, C., Slaughter, C., Cobb,

M. H., and Shaw, P. E. (1995) *EMBO J.* **14,** 951–962
52. Dalton, S. and Treisman, R. (1992) *Cell* **68,** 597–612
53. Hipskind, R. A., Rao, V. N., Mueller, C. G., Reddy, E. S., and Nordheim, A. (1991) *Nature* **354,** 531–534
54. Whitmarsh, A. J., Shore, P., Sharrocks, A. D., and Davis, R. J. (1995) *Science* **269,** 403–407
55. Brunner, D., Ducker, K., Oellers, N., Hafen, E., Scholz, H., and Klambt, C. (1994) *Nature* **370,** 386–389
56. Jiang, W., Kahn, S. M., Zhou, P., Zhang, Y. J., Cacace, A. M., Infante, A. S., Doi, S., Santella, R. M., and Weinstein, I. B. (1993) *Oncogene* **8,** 3447–3457
57. Quelle, D. E., Ashmun, R. A., Shurtleff, S. A., Kato, J. Y., Bar-Sagi, D., Roussel, M. F., and Sherr, C. J. (1993) *Genes Dev.* **7,** 1559–1571
58. Resnitzky, D., Gossen, M., Bujard, H., and Reed, S. I. (1994) *Mol. Cell. Biol.* **14,** 1669–1679
59. Lukas, J., Bartkova, J., Welcker, M., Petersen, O. W., Peters, G., Strauss, M., and Bartek, J. (1995) *Oncogene* **10,** 2125–2134
60. Mizukami, Y., Hirata, T., and Yoshida, K. (1997) *FEBS Lett.* **401,** 247–251
61. Yoshida, K., Hirata, T., Akita, Y., Mizukami, Y., Yamaguchi, K., Sorimachi, Y., Ishihara, T., and Kawashima, S. (1996) *Biochim. Biophys. Acta* **1317,** 36–44
62. Yoshida, K. and Nachmias, V. T. (1987) *J. Biol. Chem.* **262,** 16048–16054
63. Yoshida, K., Stark, F., and Nachmias, V. T. (1988) *Biochem. J.* **249,** 487–493
64. Bokemeyer, D., Guglielmi, K. E., McGinty, A., Sorokin, A., Lianos, E. A., and Dunn, M. J. (1997) *J. Clin. Invest.* **100,** 582–588
65. Floege, J., Eng, E., Young, B. A., Alpers, C. E., Barrett, T. B., Bowen-Pope, D. F., and Johnson, R. J. (1993) *J. Clin. Invest.* **92,** 2952–2962
66. Purkerson, M. L., Tollefsen, D. M., and Klahr, S. (1988) *J. Clin. Invest.* **81,** 69–74
67. Suzuki, H., O'Neill, B. C., Suzuki, Y., Denisenko, O. N., and Bomsztyk, K. (1996) *J. Biol. Chem.* **271,** 18981–18988
68. Border, W. A., Okuda, S., Languino, L. R., Sporn, M. B., and Ruoslahti, E. (1990) *Nature* **346,** 371–374
69. Isaka, Y., Fujiwara, Y., Ueda, N., Kaneda, Y., Kamada, T., and Imai, E. (1993) *J. Clin. Invest.* **92,** 2597–2601
70. Kim, S.-J., Angel, P., Lafyatis, R., Hattori, K., Kim, K. Y., Sporn, M. B., Karin, M., and Roberts, A. B. (1990) *Mol. Cell. Biol.* **10,** 1492–1497
71. Martin, M., Vozenin, M.-C., Gault, N., Crechet, F., Pfarr, C. M., and Lefaix, J.-L. (1997) *Oncogene* **15,** 981–989
72. Kim, S.-J., Denhez, F., Kim, K. Y., Holt, J. T., Sporn, M. B., and Roberts, A. B. (1989) *J. Biol. Chem.* **264,** 19373–19378
73. Mucsi, I., Skorecki, K. L., and Goldberg, H. J. (1996) *J. Biol. Chem.* **271,** 16567–16572