

Cyclic Strain Stress-induced Mitogen-activated Protein Kinase (MAPK) Phosphatase 1 Expression in Vascular Smooth Muscle Cells Is Regulated by Ras/Rac-MAPK Pathways*

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Recently, we demonstrated that mechanical stress results in rapid phosphorylation or activation of platelet-derived growth factor receptors in vascular smooth muscle cells (VSMCs) followed by activation of mitogen-activated protein kinases (MAPKs) and AP-1 transcription factors (Hu, Y., Bock, G., Wick, G., and Xu, Q. (1998) *FASEB J.* 12, 1135–1142). Herein, we provide evidence that VSMC responses to mechanical stress also include induction of MAPK phosphatase-1 (MKP-1), which may serve as a negative regulator of MAPK signaling pathways. When rat VSMCs cultivated on a flexible membrane were subjected to cyclic strain stress (60 cycles/min, 5–30% elongation), induction of MKP-1 proteins and mRNA was observed in time- and strength-dependent manners. Concomitantly, mechanical forces evoked rapid and transient activation of all three members of MAPKs, *i.e.* extracellular signal-regulated kinases (ERKs), c-Jun NH₂-terminal protein kinases (JNKs), or stress-activated protein kinases (SAPKs), and p38 MAPKs. Suramin, a growth factor receptor antagonist, completely abolished ERK activation, significantly blocked MKP-1 expression, but not JNK/SAPK and p38 MAPK activation, in response to mechanical stress. Interestingly, VSMC lines stably expressing dominant negative Ras (Ras N17) or Rac (Rac N17) exhibited a marked decrease in MKP-1 expression; the inhibition of ERK kinases (MEK1/2) by PD 98059 or of p38 MAPKs by SB 202190 resulted in a down-regulation of MKP-1 induction. Furthermore, overexpressing MKP-1 in VSMCs led to the dephosphorylation and inactivation of ERKs, JNKs/SAPKs, and p38 MAPKs and inhibition of DNA synthesis. Taken together, our findings demonstrate that mechanical stress induces MKP-1 expression regulated by two signal pathways, including growth factor receptor-Ras-ERK and Rac-JNK/SAPK or p38 MAPK, and that MKP-1 inhibits VSMC proliferation via MAPK inactivation. These results suggest that MKP-1 plays a crucial role in mechanical stress-stimulated signaling leading to VSMC growth and differentiation.

Intracellular signaling stimulated by growth factors, cytokines, osmotic shock, and stress involves the initiation of one or more phosphorylation cascades leading to the rapid and revers-

ible activation of mitogen-activated protein kinases (MAPKs),¹ a family of ubiquitous and well characterized serine/threonine kinases thought to play a critical role in regulating cellular events required for cell growth, differentiation, and apoptosis (1–3). Three major subfamilies of MAPKs have been identified, including the extracellular signal-regulated kinases (ERKs), c-Jun NH₂-terminal protein kinases (JNKs) or stress-activated protein kinases (SAPKs), and p38 MAPKs (1–3). They are strongly activated in the arterial wall in response to angioplasty (4–7), hypertension (8), and hypercholesterolemia,² which are risk factors for vascular diseases.

MAPK phosphatase-1 (MKP-1) has dual catalytic activity toward phosphotyrosine- and phosphothreonine-containing proteins and is known to inactivate ERKs and possibly JNKs/SAPKs (9–12), which play an important role in the regulation of mitogenesis. MKP-1, regulated at the transcriptional level, is induced in vascular smooth muscle cells (VSMCs) by growth factors (13), oxidative stress (14), arachidonic acid (15), and 12-*O*-tetradecanoylphorbol-13-acetate (16). Although MKP-1 has been implicated in a feedback loop that inactivates MAPKs after stimulation by mitogens and during the cellular response to stress (10, 12, 17, 18), signal pathways leading to MKP-1 gene expression are not fully elucidated.

In vivo, vessel walls are exposed to three main hemodynamic forces: shear stress, the dragging frictional force created by blood flow; transmural pressure, created by the hydrostatic forces of blood within the blood vessel; and mechanical stretch or tension, a cyclic strain stress created by blood pressure (19, 20). VSMCs are one of the major constituents of blood vessel wall responsible for the maintenance of vascular tone (21). Factors ranging from physical exertion to psychological stress lead to a transient rise in blood pressure (22, 23), and if the factors are persistent and chronic, the arteriole walls gradually thicken, resulting in hypertension (22–25). In humans, atherosclerotic lesions occur preferentially at bifurcations and curvatures (26), where hemodynamic force is disturbed (27). There is growing evidence that mechanical force initiates intracellular signaling and regulates the synthesis and/or secretion of numerous factors, including NO (28), prostacyclin (29), endothelin-1 (30), platelet-derived growth factor, fibroblast growth factor (31, 32), and angiotensin II (33, 34), which are crucial factors in maintaining the homeostasis of the vessel wall. Thus, mechanical stress plays an important role in the development of hypertension and atherosclerosis (35).

Xu *et al.* (36) have previously shown that acute hypertension

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¹ The abbreviations used are: MAPK(s), mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; GST, glutathione *S*-transferase; JNK, c-Jun NH₂-terminal protein kinase; MKP, MAPK phosphatase; rMKP, rat MKP; SAPK, stress-activated protein kinase; VSMC, vascular smooth muscle cell; Mops, 4-morpholinepropanesulfonic acid.

² B. Metzler, Y. Hu, H. Dietrich, and Q. Xu, unpublished data.

induces a rapid and transient expression of MKP-1 mRNA followed by elevated MKP-1 protein in rat aorta. The MKP-1 induction is blocked by prevention of elevation in blood pressure, *i.e.* administration of the vasodilator agent sodium nitroprusside. However, it is not known whether MKP-1 production is initiated by hemodynamic force *per se* or by hormones, cytokines *in vivo*. In the present study, we evaluated potential effects of mechanical stress on MKP-1 induction in VSMCs cultivated on a flexible membrane and subjected to cyclic strain stress. We demonstrated that mechanical stress causes rapid MKP-1 expression in VSMCs, which appears to be mediated by Ras- and Rac-MAPK pathways, respectively.

EXPERIMENTAL PROCEDURES

Materials—A rat MKP-1 cDNA was isolated from rat lung cDNA library by Liu *et al.* (11). Polyclonal antibodies against MKP-1, ERK1/2, JNK/SAPK, and mouse monoclonal antibodies against phosphorylated-ERK1/2, -JNK1/2, and -p38 MAPK and against Ha-Ras were obtained from Santa Cruz Biotech., Santa Cruz, CA. Suramin and G418 were obtained from Sigma. PD 98059 and SB 202190 were purchased from Calbiochem-Novabiochem. Plasmids expressing dominant negative Ras (Ras N17) and dominant negative Rac1 (Rac1 N17) and myc-tagged antibody were provided by G. Baier (Institute for Medical Biology and Human Genetics, University of Innsbruck, Austria). SuperFect reagent for transfection was purchased from Qiagen (Valencia, CA).

Cell Culture—VSMCs were isolated by enzymatic digestion of rat aortas using a modification of the procedure of Ross and Kariya (37), as described previously (38), and cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 20% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 µg/ml). Cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂. The medium was changed every 3 days, and cells were passaged by treatment with 0.2% trypsin, 0.02% EDTA solution. Experiments were conducted on VSMCs that had just achieved confluence.

Stable Transfection—Rat VSMCs were transfected with Ras N17, Rac N17, and MKP-1 plasmids, respectively, by using SuperFect Kit according to the manufacturer's instructions. After transfection, the cells were cultured for 24 h, divided 1 to 4, and placed in culture medium supplemented with 20% fetal calf serum and 150 µg/ml G418 to select those carrying a neomycin-resistant plasmid. When 80% cell death in a parallel group of normal VSMCs was observed, the medium containing 150 µg/ml G418 was changed into medium containing 50 µg/ml G418 to maintain selection. After 4–8 weeks, individual cell colonies were transferred for clone expansion and maintained in culture medium supplemented with 20% fetal calf serum and 50 µg/ml G418. Ras N17-, Rac N17-, and MKP-1-transfected VSMCs were identified by Western blotting analysis with antibodies to Ha-Ras, myc-tagged, and MKP-1 proteins.

Cyclic Strain Stress—VSMCs were plated on silicone elastomer-bottomed culture plates (Flexcell, Meckesport, PA). Cells achieving 90% confluence were serum-starved for 3 days and subjected to mechanical stress with the Cyclic Stress Unit, a modification of the unit initially described by Baner *et al.* (39) consisting of a controlled vacuum unit and a base plate to hold the culture plates (FX3000 AFC-CTL, Flexcell). A vacuum (15 to 20 kilopascals) was repeatedly applied to the elastomer-bottomed plates via the base plate, which was placed in a humidified incubator with 5% CO₂ at 37 °C. Cyclic deformation (60 cycles/min) and 5 to 30% elongation of elastomer-bottomed plates were used (40).

Cell Pretreatment—VSMCs, growing in silicone elastomer-bottomed culture plates to 90% confluence, were serum-starved for 3 days, and suramin, PD 98059, or SB 202190 was added and incubated for 1 h before application of cyclic strain stress.

Protein Extraction and Western Blot Analysis—After strain stress, VSMCs were washed twice with cold (4 °C) phosphate-buffered saline (pH 7.4) and harvested on ice in buffer A containing 20 mM Hepes (pH 7.4), 2 mM EDTA, 50 mM β-glycerophosphate, 1 mM dithiothreitol, 1 mM Na₃VO₄, 1% Triton, 10% glycerol, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 100 µM phenylmethylsulfonyl fluoride. The suspension was incubated on ice for 20 min with vortexing every 5 min. Cellular debris were then pelleted by centrifugation for 30 min at 13,000 rpm (Eppendorf centrifuge, Osterode, Germany) at 4 °C, supernatants were collected, and protein concentration measured by the Bio-Rad assay.

Membrane protein preparation for Ras and Rac analysis was similar to that described by Pomerantz *et al.* (41). Briefly, VSMCs were washed with cold phosphate-buffered saline (4 °C), scraped in phosphate-buffered saline, pelleted, and resuspended in 500 µl of homogenizing buffer

(25 mM Hepes, 1.0 mM EDTA, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, and 100 µM phenylmethylsulfonyl fluoride). Cells were sonicated for 10 s and centrifuged at 2,000 rpm for 10 min to remove debris. The supernatant was centrifuged at 55,000 × *g* for 1 h at 4 °C. The cytosolic supernatant was removed, the membrane pellet was resuspended in 50 µl of homogenizing buffer and sonicated for 10 s, and protein concentration was measured. For total protein extract, cells were washed with cold phosphate-buffered saline (4 °C), pelleted, resuspended in 100 µl of buffer A, sonicated for 10 s, and centrifuged at 2,000 rpm for 10 min before supernatant harvest. For Ras and Rac protein analysis, 30–100 µg of membrane protein was used in reduced conditions (2.5% SDS, 250 µM dithiothreitol for 5 min at 90 °C). The procedure used for Western blot analysis was similar to that described previously (42). In short, 30–100 µg of proteins were separated by electrophoresis through a 10% or 12% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes. The blots were probed with antibodies against MKP-1, Ha-Ras, myc-tagged Rac1, phosphorylated-ERK1/2, -JNK1/2, or -p38 MAPK, and specific antibody-antigen complexes were detected using the ECL Western blot Detection Kit (Amersham Pharmacia Biotech). Graphs of blots were obtained in the linear range of detection and were quantified for the level of specific induction by scanning laser densitometry (Power-Look II, UMAX Data System Inc., Hsinchu, Taiwan) of graphs.

RNA Isolation and Northern Blot—Total RNA was isolated as described elsewhere (36). RNA (10 µg/lane) was denatured with formaldehyde (Merck), electrophoresed in a 1% agarose gel, transferred onto nylon membrane (Zeta Probe, Bio-Rad), and UV-cross-linked in a UV Stratalinker (Stratagene Inc., La Jolla, CA). Hybridizations were performed using a fluorescein-labeled (Amersham Pharmacia Biotech) cDNA probe for MKP-1, as described previously (36). The membranes were then washed, detected with anti-fluorescein alkaline phosphatase conjugate (1:5,000) (Amersham Pharmacia Biotech), and exposed to ECL films. Graphs of blots were obtained in the linear range of detection. Accuracy of loading and transfer as well as RNA integrity was confirmed by quantitative analysis of the 28 S and 18 S RNA.

Kinase Assays—For kinase assays, 0.5 ml of supernatant containing 0.5 mg of proteins were incubated with 10 µl of antibodies against mammalian ERK2, JNK/SAPK, or p38 MAPKs for 2 h at 4 °C with rotation. Subsequently, 40 µl of protein G-agarose suspension (Santa Cruz Biotech. Inc.) was added and rotation was continued for 1 h at 4 °C. Immunocomplexes were precipitated by centrifugation and washed twice with buffers A, B (500 mM LiCl, 100 mM Tris, 1 mM dithiothreitol, 0.1% Triton X-100, pH 7.6), and C (20 mM Mops, 2 mM EGTA, 10 mM MgCl₂, 1 mM dithiothreitol, 0.1% Triton X-100, pH 7.2), respectively.

ERK2 or p38 activities in the immunocomplexes were measured as described previously (43, 44). Briefly, immunocomplexes were incubated with myelin basic protein (6 µg; Upstate Biotechnology, Inc., Lake Placid, NY) and [³²P]ATP (5 µCi) for 20 min. To stop the reaction, 15 µl of 4× Laemmli buffer was added, and the mixture was boiled for 5 min. Proteins in the kinase reaction were resolved by SDS-polyacrylamide gel electrophoresis (15% gel) and subjected to autoradiography.

The assay for JNK/SAPK activity was performed as described above using GST-c-Jun as a substrate (the plasmid was provided by Dr. J. Woodgett) produced in *Escherichia coli* and isolated using glutathione Sepharose 4B RediPack columns (Amersham Pharmacia Biotech) per manufacturer's protocol. Proteins in the kinase reaction were resolved by SDS-polyacrylamide gel electrophoresis (12% gel) and subjected to autoradiography (3, 43, 44).

[³H]Thymidine Incorporation—Transfected VSMCs cultured in the flexible plates in medium containing 20% fetal calf serum at 37 °C for 24 h were serum-starved for 4 days. VSMCs were stressed by elongation of 15% for 30 min (60 cycles/min) and incubated at 37 °C for 24 h. [³H]Thymidine was added 4 h before cell harvest. Radiation activities were measured.

Statistical Analysis—Analysis of variance was performed when more than two groups were compared. An unpaired Student's *t* test was used to assess differences between two groups. A *p* value less than 0.05 was considered significant.

RESULTS

Cyclic Strain Stress Induced MKP-1 Expression—To explore the possibility that the expression of MKP-1 is altered after mechanical stress, MKP-1 mRNA in stressed VSMCs were determined by Northern blot analysis. As shown in Fig. 1A, strain stress treatment (60 cycles/min, 15% elongation) resulted in significant increases in MKP-1 mRNA. Kinetic anal-

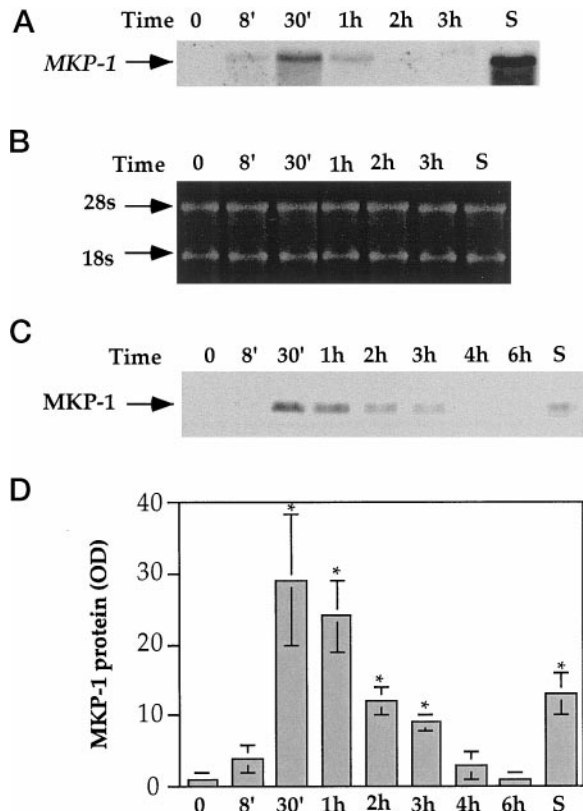


FIG. 1. Time course of MKP-1 expression in VSMCs exposed to mechanical stress. Serum-starved VSMCs were treated with cyclic strain stress (60 cycles/min, 15% elongation) for the indicated times and harvested. *Panel A*, Northern blot analysis. Total RNA was extracted from VSMCs, and 10 μ g of total RNA/lane were electrophoresed on 1% agarose and transferred to membrane. The integrity and quantity of RNA were verified by analysis of 18 S and 28 S RNA (*panel B*). Northern blots were hybridized with MKP-1 cDNA probes. Data are representative of two independent experiments. *Panel C*, Western blot analysis. After treatment, cells were lysed in the buffer A, and protein extracts were separated on 10% SDS-polyacrylamide gel, transferred to membranes, and probed using an antibody to MKP-1 protein. *Panel D* shows statistical data of MKP-1 protein induction from VSMCs treated with mechanical stress. *, significant difference from control, $p < 0.05$. S, fetal calf serum treatment as a positive control.

ysis indicates that this response occurred as early as 8 min with maximum induction achieved 30 min after treatment and declining thereafter (Fig. 1A). Fig. 1B showed 18 S and 28 S RNA from the corresponding blot, indicating a similar amount of RNA loaded. Likewise, growth-arrested VSMCs were exposed to cyclic strain stress for various times, and protein extracts from control and treated cells were analyzed for MKP-1 induction. As shown in Fig. 1C, cells treated with mechanical stress resulted in a time-dependent induction of MKP-1 proteins that was evident at 8 min, peaked at 30 min, and returned to basal line by 4 h. Fig. 1D summarizes data of MKP-1 protein induction as determined by quantification of optical densities from autoradiograms of three experiments. A significant increase in MKP-1 proteins of VSMCs was observed between 30 min and 3 h.

To further establish the relationship between mechanical strain stress and MKP-1 expression, a tensile strength response analysis of mechanical stress-induced MKP-1 mRNA accumulation was performed. As shown in Fig. 2A, VSMCs were stretched for elongation of 5, 10, 15, 20, 25, and 30% of original size, respectively, and the increase of MKP-1 mRNA amounts corresponded with the increased magnitudes of stretch stress of 5–20%. Fig. 2B shows the amount of 18 S and 28 S RNA from the corresponding blot. Similar results were

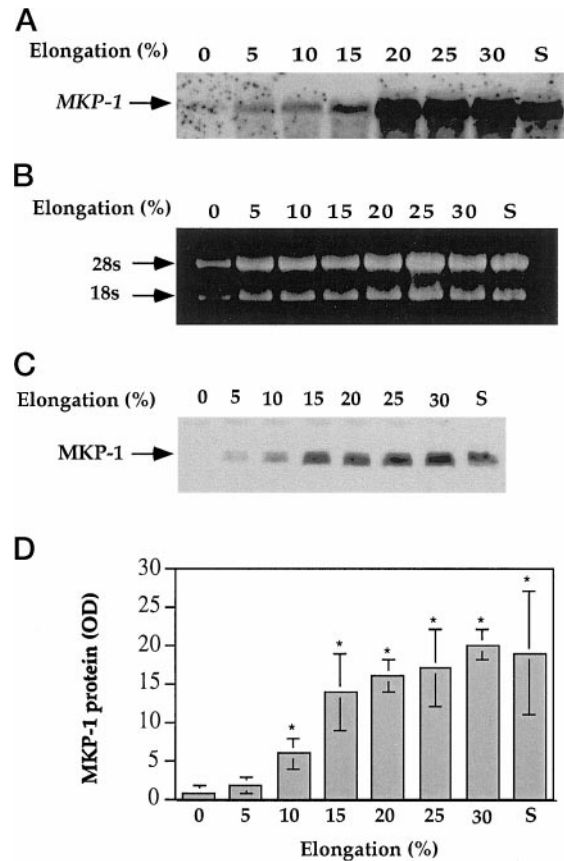


FIG. 2. Effects of mechanical stress strength on MKP-1 induction. VSMCs were serum-starved for 3 days and treated with strain stress (60 cycles/min, 5–30% elongation) for 30 min. Northern blots (*panels A and B*) and Western blot (*panel C*) were performed as described in the legend to Fig. 1 and under “Experimental Procedures.” *Panel D* summarizes the results of Western blots of three independent experiments. *, significant difference from control, $p < 0.05$. S, fetal calf serum treatment as a positive control.

observed at the level of MKP-1 proteins (Fig. 2C). Fig. 2D shows statistical data from three experiments. A significant induction was found in stressed VSMCs elongated between 10 and 30%. Our results provided the first evidence that MKP-1 is induced by mechanical stress in VSMCs.

There is evidence that mechanical force rapidly activates ERK and JNK/SAPK in VSMCs (45, 46), but no data exists as to whether p38 MAPK is also activated in response to cyclic strain stress. Phosphorylation of p38 MAPK, ERK1/2, and JNK1/2 was determined by Western blot analysis using anti-phosphorylated-p38 MAPK, -ERK1/2, and -JNK1/2 antibodies, respectively. Fig. 3A shows phosphorylation of p38 MAPK in VSMCs treated with cyclic strain stress for various times. The highest level of p38 phosphorylation was obtained between 8 and 30 min and declined thereafter. Similarly, ERK 1/2 phosphorylation was evident, but ERK proteins did not alter in response to strain stress (Fig. 3, B and C). ERK activity was measured for immunoprecipitated ERK2 to phosphorylate myelin basic protein, indicating that mechanical strain stress induced marked ERK activation (Fig. 3D). Strain stress also resulted in a rapid activation of JNK/SAPK (Fig. 3, E and F) in VSMCs. The activities of three MAPKs declined between 30 and 120 min in response to mechanical stress (Fig. 3), whereas higher levels of MKP-1 proteins persisted (Fig. 1C), suggesting a role of MKP-1 on inactivation of MAPKs.

Suramin Partially Blocked MKP-1 Expression—We recently demonstrated that mechanical stress directly stimulates platelet-derived growth factor receptor phosphorylation or activa-

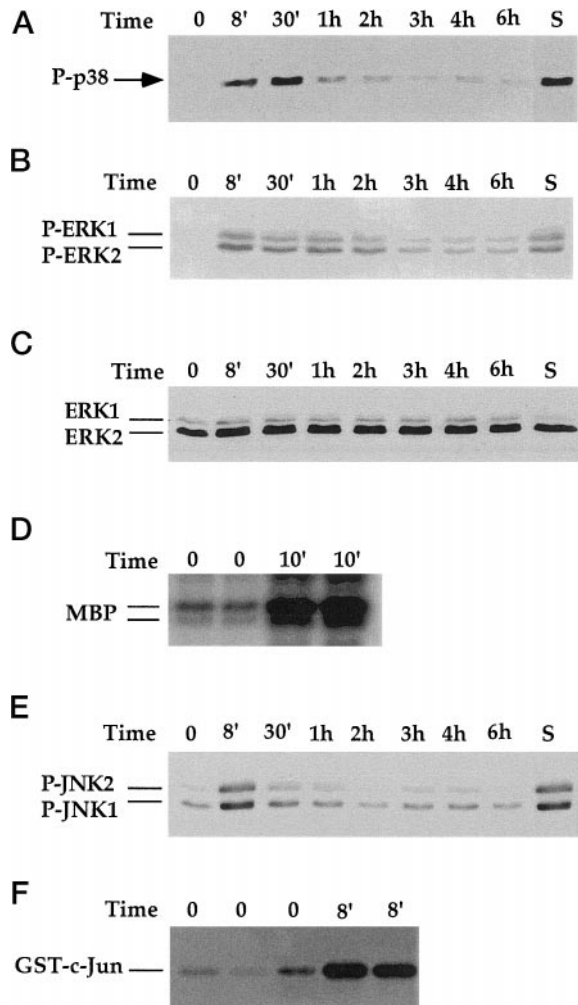


FIG. 3. Mechanical stress-induced ERK and JNK/SAPK and p38 MAPK activation. Serum-starved VSMCs were treated with strain stress (60 cycles/min, 15% elongation) for the indicated times and harvested for protein extracts. The results of Western blot analysis were shown for phosphorylated (P) p38 (panel A), ERK1/2 (panel B), and JNK1/2 (panel E). For the kinase assay, ERK2 and JNK1 proteins were immunoprecipitated from the protein extracts, and their kinase activities were measured based on phosphorylation of myelin basic protein (MBP) substrate (panel D) and GST-c-Jun fusion protein substrate (panel F). Panel C shows the total ERK1/2 proteins labeled with anti-pan-ERK1/2 antibodies for each sample tested. Data represent similar results from three independent experiments. S, fetal calf serum treatment as positive controls.

tion and that suramin, a broad spectrum receptor antagonist, blocked such activation (40). It was not known if growth factor receptors were also responsible for MKP-1 induction during mechanical stress. Suramin treatment markedly blocked MKP-1 expression (Fig. 4A), suggesting that the initial signal in mechanically stressed-VSMCs is, at least in part, generated on the plasma membrane via growth factor receptor activation. As expected, suramin completely blocked mechanical stress-stimulated phosphorylation of ERK (Fig. 4B) but not JNK/SAPK (Fig. 4C). Surprisingly, it apparently enhanced the activation of p38 MAPK (Fig. 4D). These results suggest that suramin-blocked MKP-1 expression may be due to inhibition of the growth factor receptor-ERK pathway, independent of the JNK/SAPK and P38 MAPK pathways.

Involvement of Ras/Rac in MKP-1 Expression—A large number of extracellular signals stimulates Ras activation, resulting in Ras-dependent MAPK activation. Recent data reported by Gudi *et al.* (47) indicate that Ras protein was activated in response to shear stress in endothelial cells. It would be inter-

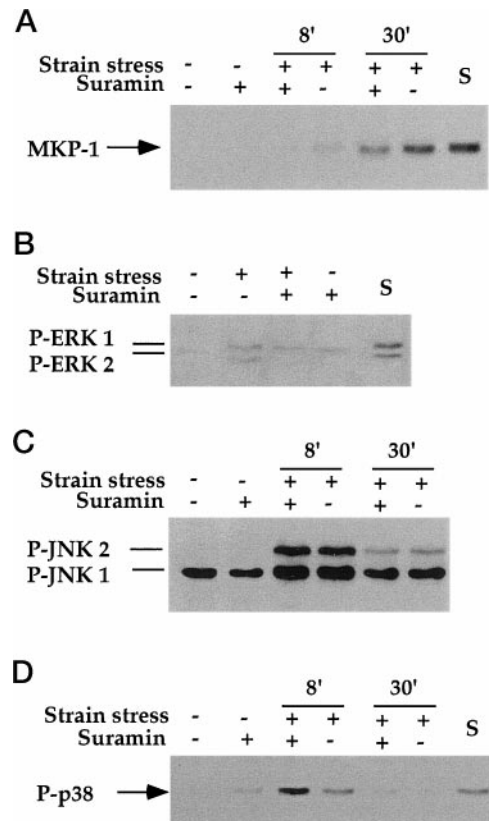


FIG. 4. Effects of suramin on MKP-1 expression. Quiescent VSMCs were pretreated with suramin (0.3 mM) for 30 min. Cells were treated with cyclic strain stress for 8 or 30 min, respectively, and harvested for protein extracts. The results of Western blot analysis are shown for MKP-1 (panel A), phosphorylated (P) ERK1/2 (panel B), JNK1/2 (panel C), p38 MAPK (panel D). The experimental procedures are similar to those described in the legend to Fig. 1. Data are representative of similar results of three independent experiments. S, fetal calf serum treatment as positive controls.

esting to determine whether Ras also mediates MKP-1 expression in stressed VSMCs. VSMCs were stably transfected with plasmid-expressing dominant negative Ras (Ras N17) or a vector plasmid as control. Since plasmids contain the selective marker gene *neo*, multiple G418-resistant colonies were selected and tested for expression of Ras protein by Western blotting analysis with anti-Ha-Ras antibody. As shown in Fig. 5A, Ras protein was at a lower level in vector-transfected controls and much higher in Ras-transfected cells. Ras N17 expression reduced ERK activation (Fig. 5B) and significantly blocked MKP-1 induction (Fig. 5, C and D), indicating Ras-ERK-dependent MKP-1 induction. Interestingly, stronger stress (25% elongation)-induced MKP-1 expression can only be partially blocked by Ras N17, suggesting that other signal pathways may be also involved.

Rac is a member of the Ras superfamily of small GTP-binding proteins. Increasing evidence indicates that members of Rac regulate a diverse array of cellular events, including the control of cell growth, cytoskeletal reorganization and the activation of protein kinases, and cardiac myocyte hypertrophy (48). To explore the role of Rac in MKP-1 expression in stress-stimulated VSMCs, we established VSMC lines stably expressing Rac1, encoding a myc-tagged form of a dominant negative Rac1 (Rac1 N17) that expressed a high level of this gene product (Fig. 6A). Surprisingly, overexpression of Rac1 N17 markedly inhibited ERK1/2 phosphorylation (Fig. 6B), indicating Rac-dependent ERK activation in response to mechanical stress. We next assessed the effects on MKP-1 expression in the Rac1 N17 cell lines treated with mechanical stress. As seen in

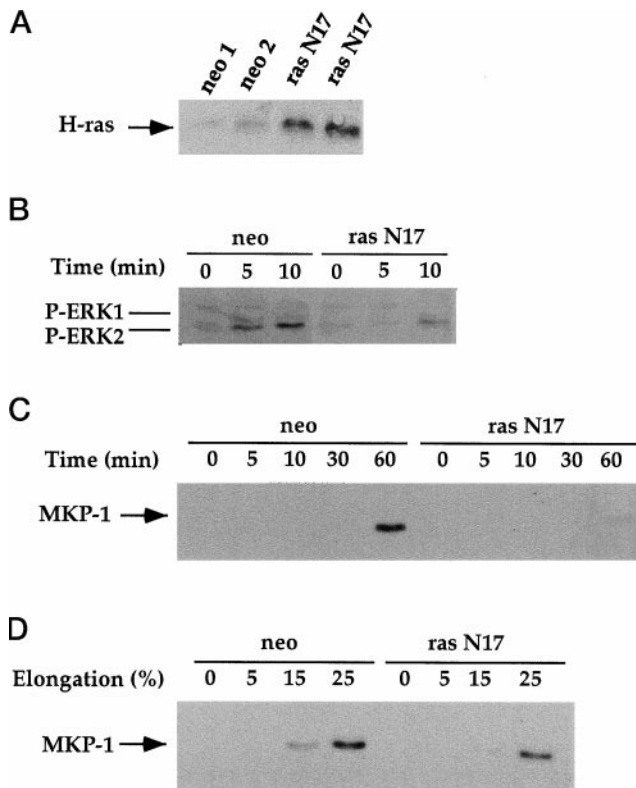


FIG. 5. Involvement of Ras in MKP-1 expression. Rat VSMCs were stably transfected with constructs expressing dominant negative Ras (pEF-Ras N17) or vector (pEF-neo) using the SuperFect reagent in a ratio of 1 to 2 (w/w). The transfected cells were cultured overnight, divided 1 to 4, and placed in culture medium supplemented with 150 μ g/ml G418. VSMC lines expressing Ras N17 were identified with anti-Ha-Ras (*H-Ras*) antibody, as determined by Western blotting (*panel A*). VSMC lines were serum-starved for 3 days and treated with mechanical stress for the indicated times (60 cycles/min, 15% elongation) (*panels B and C*) or the indicated elongations for 1 h (*panel D*). Western blot analysis was performed using anti-phosphorylated (*P*) ERK1/2 (*B*) or anti-MKP-1 (*C and D*) antibodies. Data represent similar results from three independent experiments.

Fig. 6, *C and D*, MKP-1 expression in Rac1 N17 cell lines was much lower than in vector-transfected cell lines. The results suggest that Rac plays an important role in the signal transduction leading to MKP-1 expression in VSMCs.

ERK- and p38-dependent MKP-1 Induction—As shown in Figs. 1 to 3, ERK, JNK, and p38 MAPK activation by mechanical stress proceeded to MKP-1 expression. To investigate whether these kinases are involved in strain stress-stimulated MKP-1 expression, ERK, JNK, and p38 MAPK phosphorylation and MKP-1 expression were simultaneously determined in stressed VSMCs treated with PD 98059, a specific MAPK kinase (MEK1/2) inhibitor. Mechanical stress-induced ERK1/2 phosphorylation was completely abolished by PD 98059 pretreatment (Fig. 7*A*) but not JNKs/SAPKs (Fig. 7*B*) and p38 MAPKs (Fig. 7*C*). MKP-1 induction was evidently inhibited (Fig. 7*D*). These results support the notion that mechanical stress-induced MKP-1 expression is partially dependent on ERK activation. Furthermore, p38 MAPK activity was abrogated by SB 202190, a specific inhibitor for p38 MAPKs (Fig. 8*A*). It was unexpected that mechanical stress-induced MKP-1 expression was also inhibited, at least in part (Fig. 8*B*). These observations implicate the involvement of p38 MAPKs in the MKP-1 induction.

MKP-1 Inhibited MAPK Activation and VSMC Proliferation—To study the effects of MKP-1 on MAPK activation and on VSMC proliferation, we established VSMC lines overexpressing MKP-1 and determined ERK, JNK/SAPK, and p38

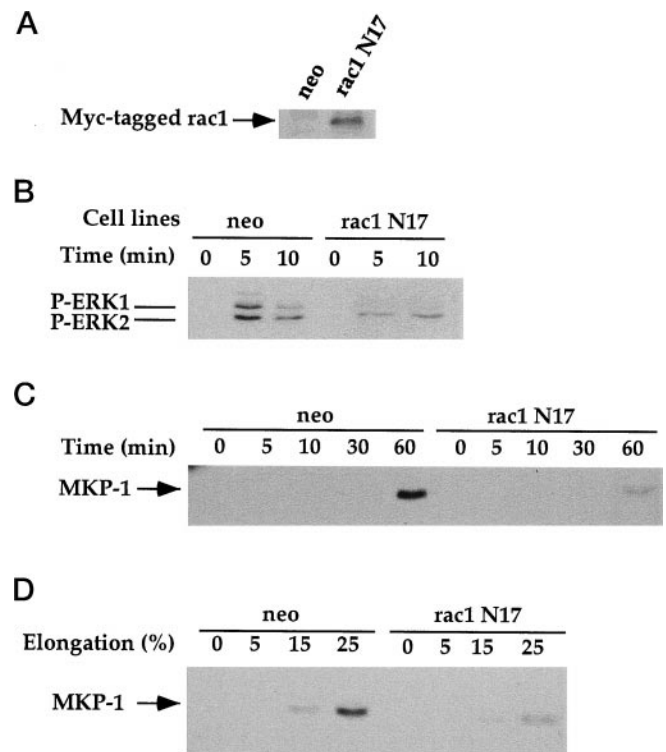


FIG. 6. Involvement of Rac in MKP-1 expression. The procedures for establishing stably expressing dominant negative Rac1 (pEF-Rac1 N17) cell lines are similar to those described in the legend to Fig. 5 and under "Experimental Procedures." *Panel A* shows the results of Western blot analysis using anti-myc-tag antibody. VSMC cell lines were serum-starved for 3 days and treated with mechanical stress for the indicated times (15% elongation) (*Panel C*) and the indicated elongations for 1 h (*panel D*). Western blot analysis was performed using anti-phosphorylated (*P*) ERK1/2 (*B*) or anti-MKP-1 (*C and D*) antibodies. Data represent similar results from three independent experiments.

MAPK phosphorylation and DNA synthesis in response to mechanical stress. Transfected clones stably expressing MKP-1 were identified by Western blot analysis. Although our antibody could not differentiate endogenous rat MKP-1 from overexpressed rMKP-1 proteins, the cells under low growth conditions (2% calf serum or 5% stretch elongation) minimized endogenous MKP-1 to an undetectable level, as seen in the Western blot (Fig. 9*A*). Cells transfected with MKP-1-expressing plasmids showed a high level of MKP-1 (Fig. 9*A*). Compared with vector-transfected cells, a 50–80% reduction in ERK, JNK/SAPK, and p38 MAPK phosphorylation was observed in MKP-1-expressing VSMCs when stimulated with mechanical stress (Fig. 9, *B–D*). To determine the effects of MKP-1 on DNA synthesis induced by mechanical stress, rMKP-1 or vector-transfected cell lines were stimulated by mechanical stress for 30 min. As shown in Fig. 9*E*, [3 H]thymidine incorporation in rMKP-1-expressing VSMCs was significantly lower than vector-transfected cells. These results suggest that MKP-1 inhibits VSMC growth via inactivation or dephosphorylation of three MAPKs.

DISCUSSION

Recent evidence indicates that mechanical stress is an important extracellular stimulus and regulates gene expression, protein synthesis, and growth and differentiation of cardiovascular cells (28–34, 45–55). The present study demonstrates for the first time that mechanical stress causes MKP-1 expression, which is crucial in regulation of MAPK activities in VSMCs. Mechanical stress also induces activation of ERK1/2, JNKs/SAPKs, and p38 MAPKs. This process involves small molecular GTP-binding proteins, Ras and Rac. These results have several

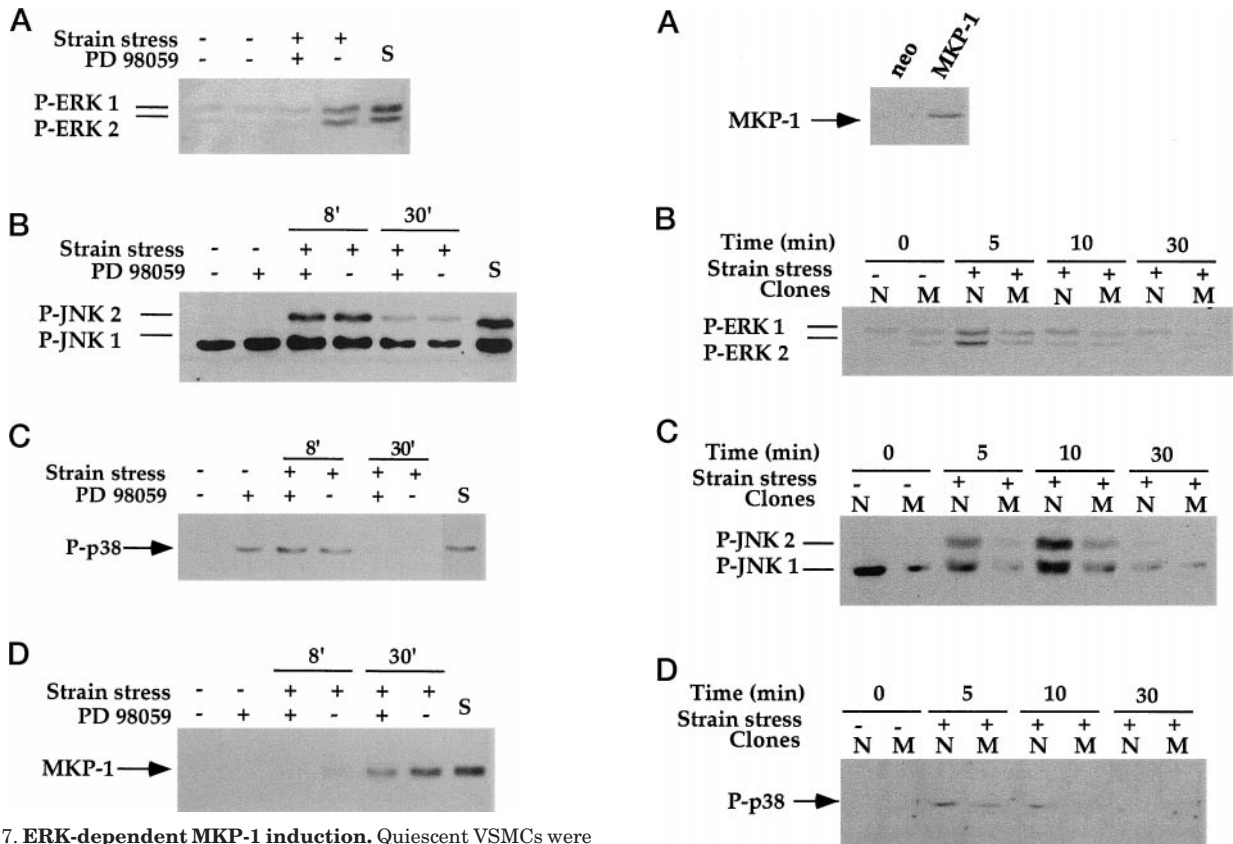


FIG. 7. ERK-dependent MKP-1 induction. Quiescent VSMCs were pretreated with PD 98059 (50 μ M) for 1 h. Cells were stressed for 8 or 30 min, respectively, and harvested for protein extracts. The results of Western blot analysis were shown for phosphorylated (P) ERK1/2 (panel A), JNK1/2 (panel B), p38 MAPK (panel C), and MKP-1 (panel D). The procedures are similar to those described in the legend to Fig. 3. Data represent similar results from three independent experiments. S, fetal calf serum treatment as positive controls.

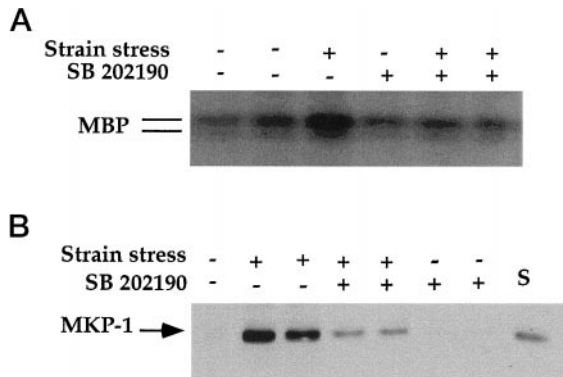


FIG. 8. p38 MAPK-dependent MKP-1 induction. Quiescent VSMCs were pretreated with SB 202190 (5 μ M) for 1 h. Cells were stressed for 8 or 30 min, respectively, and harvested for protein extracts. p38 MAPK activities were measured based on phosphorylation of myelin basic protein (MBP; panel A). The results of Western blot analysis for MKP-1 were shown in panel B. Data represent similar results from two independent experiments. S, fetal calf serum treatment as a positive control.

implications. First, understanding that strain stress induces MKP-1 expression may strengthen the notion that mechanical stress plays an important role in regulating VSMC growth. Although strain stress stimulates ERK, JNK/SAPK, and p38 MAPK activation, which appears to be a component common to signaling pathways initiated by a wide range of growth-stimulating factors, including mitogens and hormones (1–3, 56–61), MKP-1 serves as a negative regulator, controlling cell growth via inactivation of three MAPKs. Second, mechanical stress

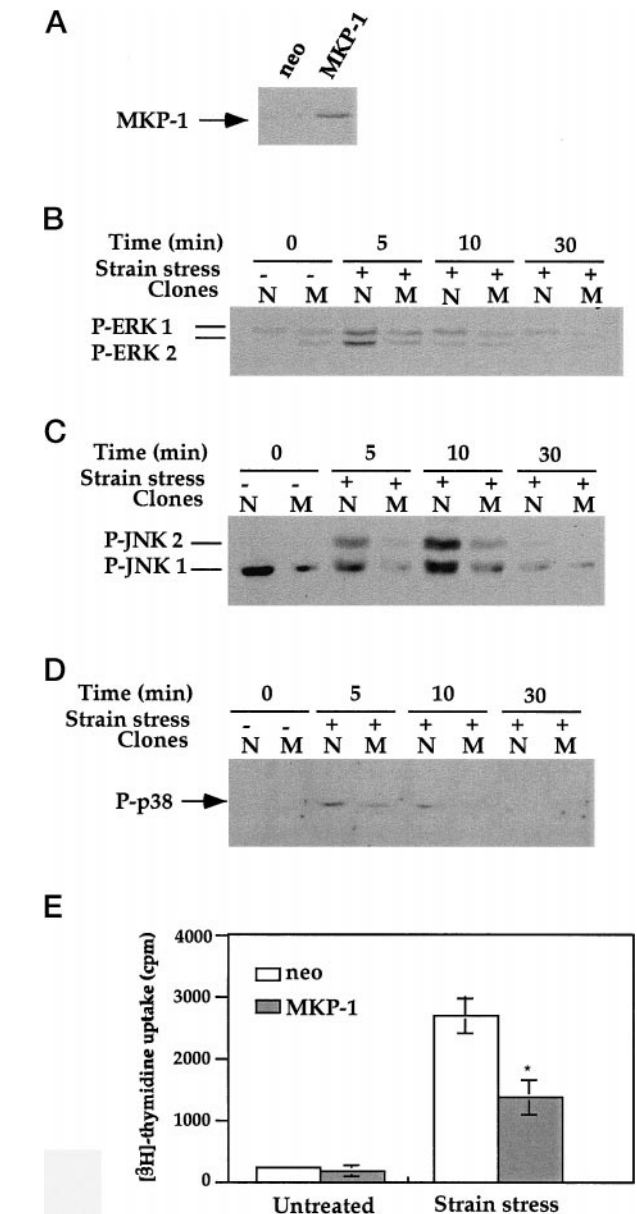


FIG. 9. MKP-1 inhibited MAPK activation and VSMC proliferation. The experimental procedures for establishing rMKP-1 (pSG5-rMKP-1) or vector (pSG5-neo)-transfected cell lines are similar to those described in the legend to Fig. 5 and under "Experimental Procedures." Panel A shows results of Western blot analysis using the anti-MKP-1 antibody. rMKP-1 or vector-transfected VSMCs were serum-starved for 3 days and treated with mechanical stress for the indicated times (60 cycles/min, 15% elongation). Western blot analysis was performed for the phosphorylated (P) ERK1/2 (panel B), JNK1/2 (panel C), and p38 MAPK (panel D). Panel E, VSMC proliferation assays. VSMC cell lines were serum-starved for 4 days and treated with mechanical stress for 30 min and incubated at 37 $^{\circ}$ C for 24 h. [3 H]Thymidine was added for 4 h before harvest. Radiation activities were measured. Data are means \pm S.D. of triplicates from two independent experiments. *, significant difference from vector-transfected cells. N, neo; M, MKP-1.

plays a crucial role in the regulation of VSMC tone caused by autocrine and paracrine vasoconstrictors (28–30, 51), such as angiotensin II and endothelin-1, which result in MKP-1 expression. In this process, MKP-1 might be involved in inactivation of numerous kinases that influence cell tone. Finally, stress-induced MKP-1 expression is not only mediated by Ras but also by Rac proteins, suggesting a complicated network of stress-induced signaling in VSMCs. Thus, our findings could significantly advance our understanding of the role of MKP-1 in VSMC proliferation, which is a key event in the pathogenesis of

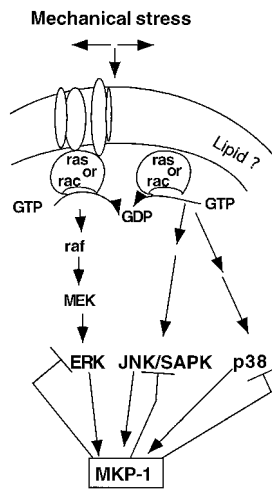


FIG. 10. Schematic representation of growth factor receptor-dependent and -independent activation of G proteins by mechanical stress. Increase in the elongational and translational mobility in membrane results in exposure of kinase domains of growth factor receptors and/or conformation of membrane-bound G proteins, which lead to receptor autophosphorylation or facilitating exchange of GDP for GTP. Subsequently, activation of MAPKs mediates MKP-1 expression, which serves as a negative regulator for MAPKs. MEK, MAPK/ERK kinase.

hypertension-related arteriosclerosis, angioplasty-induced restenosis, and vein graft arteriosclerosis.

How do the VSMCs sense and transduce the signals in response to mechanical stress, leading to MKP-1 expression? One possibility is that growth factor receptor activation is partially responsible for mechanical stress-induced MKP-1 expression. This concept is supported by our previous observation that mechanical stress could induce rapid phosphorylation of platelet-derived growth factor receptors in VSMCs (40). Suramin, a growth factor receptor antagonist, inhibited phosphorylation of platelet-derived growth factor receptor (40) and could also significantly inhibit stress-induced MKP-1 expression (Fig. 4). As we previously hypothesized (40), mechanical stress elongates and changes cellular morphology, leading to receptor conformation, resulting in exposure of the kinase domain and subsequent autophosphorylation. Thus, the mechanism of mechanical stress-induced MKP-1 expression is similar to growth factor-stimulated MKP-1 induction. Since suramin could not completely block MKP-1 expression, another possible primary mechanosensor candidate may be G proteins. Recent reports by Gudi *et al.* (47) indicate that G proteins may act as primary mechanosensors in shear-stressed endothelial cells. Treatment of endothelial cells with antisense G_{α_q} oligonucleotides inhibited shear stress-induced Ras-GTPase activity, whereas scrambled oligonucleotide treatment had no effect. Treatment of endothelial cells with pertussis toxin prevented shear stress-mediated activation of ERK1/2 (35, 47). G proteins reconstituted in liposomes, in the absence of receptors, show an increase in activity in response to shear stress (35). This assumption is supported by our results (Figs. 5 and 6) that expression of dominant negative Ras and Rac in VSMCs exposed to mechanical stress could significantly inhibit MKP-1 expression. These observations suggest that increases in the elongational and translational mobility in cell membranes activate membrane-bound G proteins by facilitating exchange of GDP to GTP, subsequently leading to MAPK activation and MKP-1 expression. Our hypothesis is schematically depicted in Fig. 10.

In our study system, we demonstrated that MAPKs play an important role in mediating mechanical stress-induced MKP-1 expression, because both PD 98059 (MEK inhibitor) and SB

202190 (p38 MAPK inhibitor) could inhibit MKP-1 expression. The time course of phosphorylation of three MAPKs appears simultaneously. Therefore, mechanical stress-induced MKP-1 expression is dependent on ERK, p38 MAPK, and possibly JNK/SAPK activation. On the other hand, MKP-1 as a negative regulator for dephosphorylation and inactivation of MAPKs has been implicated in other types of cells (9–12, 17, 18). Herein, we provided direct evidence that MKP-1 dephosphorylated three members of MAPKs in stressed VSMCs. Overexpression of MKP-1 could significantly inhibit phosphorylation and activation of MAPKs and DNA synthesis (Fig. 9), indicating important roles of MKP-1 in regulating MAPK activation and cell proliferation.

All tissues in the body are subjected to physical forces originating either from tension, created by cells themselves, or from the environment (8, 35, 62–64). The role of mechanical force as an important regulator of structure and function of mammalian cells, tissues, and organs has recently been recognized. Physical stimuli must be sensed by cells and transmitted through intracellular signal transduction pathways to the nucleus, resulting in physiological responses or pathological conditions. Growth and proliferation of VSMCs have been shown to be associated with numerous vascular disease states, including medial hypertrophy in hypertension, intimal thickening in atherosclerosis, and restenosis after angioplasty, and are believed to be related with a sustained mechanical stress (21, 27, 35). Thus, we postulate that the balance between MKP-1 and MAPK levels induced by mechanical stress in VSMCs is critical to maintain homeostasis of the arterial wall. From a therapeutic point of view, our understanding of the molecular mechanisms regulating MAPK and MAPK phosphatase activities by mechanical stress could lead to new strategies for the effective prevention and control of vascular disorders.

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