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Activation of extracellular signal-regulated kinases is essential for pressure-induced proliferation of vascular smooth muscle cells

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Abstract

In hypertension, increased transmural pressure directly influences vascular smooth muscle cells and causes cell proliferation. However, the mechanisms of transmural pressure-induced proliferation of vascular smooth muscle cells are unknown. We investigated the role of various protein kinases in pressure-induced proliferation of vascular smooth muscle cells. Pressure was applied to quiescent rat vascular smooth muscle cells in culture by compressed helium gas in a loading apparatus. Pressure application increased [^{3}H]thymidine incorporation in a time- and pressure-dependent manner and significantly increased the cell number. The pressor response was significantly suppressed by various protein kinase inhibitors for protein kinase C (bisindolylmaleimide I), tyrosine kinase (genistein), extracellular signal-regulated kinase kinase (PD98059; 2′-amino-3′-methoxyflavone) and p38 mitogen-activated protein kinases (MAPK) (SB203580; 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole). Pressure rapidly increased the phosphorylation and activity of extracellular signal-regulated kinases (ERK). Pressure also caused increment of phosphorylation level of p38 MAPK but not that of c-JUN N-terminal protein kinase (JNK). In ERK-deficient cells prepared by transfection of an antisense oligonucleotide for ERK, pressure-induced DNA synthesis was almost abolished. Our results suggest that activation of ERK is essential for pressure-induced DNA synthesis in rat vascular smooth muscle cells, in addition to activation of protein kinase C, tyrosine kinase and p38 MAPK. These processes could be involved in the pathogenesis of hypertension-related atherosclerosis.

Keywords: Cell proliferation; Extracellular signal-regulated kinase; p38 mitogen-activated protein kinase; Transmural pressure; Vascular smooth muscle cell; Signal transduction

1. Introduction

The vascular wall is constantly subjected to mechanical forces such as transmural pressure, stretch and shear stress (Chien et al., 1998; Li et al., 1998; Watase et al., 1997). Vascular smooth muscle cells are normally covered with a monolayer of endothelial cells and thus are not in direct contact with blood flow. Accordingly, vascular smooth muscle cells are mainly exposed to transmural pressure or stretch among the physiological forces. There is sufficient evidence to suggest that such mechanical forces affect vascular remodeling (Hishikawa et al., 1994; Lehoux and Tedgui, 1998). Hypertension is associated with increased transmural pressure or stretch, which directly affects vascular smooth muscle cells and results in abnormal cell proliferation (Ross, 1993), and potential progression of atherosclerosis (Ross, 1993). Although several studies have examined the role of shear stress and stretch on remodeling of the vascular system (Cheng et al., 1996; Duff et al., 1995; Li et al., 1998; Sumpio et al., 1988; Takahashi and Berk, 1998; Yamazaki et al., 1995), little is known about the mechanisms by which pure transmural pressure per se promotes proliferation of vascular smooth muscle cells.

Hishikawa et al. (1994) showed that increased transmural pressure induced DNA synthesis and cell proliferation via activation of phospholipase C and protein kinase C in rat

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vascular smooth muscle cells. The actions of many stimuli resulting in proliferative or hypertrophic growth converge on a set of cellular kinase cascades, which are collectively called the mitogen-activated protein kinase cascades (Force and Bonventre, 1998). A large body of evidence has shown that a family of mitogen-activated kinases (MAPK), such as extracellular signal-regulated protein kinases (ERK), mediates the proliferation of vascular smooth muscle cells (Force and Bonventre, 1998; Takahashi and Berk, 1998; Zou et al., 1998). Recently, Ozaki et al. (1999) reported that pressure increased activity of ERK and c-JUN N-terminal protein kinase (JNK), but not p38 MAPK in human aortic vascular smooth muscle cells. However, to what extent MAPK signaling cascades are utilized to mediate pressure-induced mitogenic response in vascular smooth muscle cells is largely unknown. Accordingly, we examined the role of MAPK in transmural pressure-induced vascular smooth muscle cell proliferation.

ERK are activated rapidly in response to a variety of extracellular stimuli such as growth factors, oxidized low-density lipoproteins (LDL) or shear stress (Brown et al., 1998; Graf et al., 1997; Kusuhara et al., 1997; Takahashi and Berk, 1996; Takahashi et al., 1997; Tseng et al., 1995; Yamakawa et al., 1998). Although it has been shown that shear stress and stretch activate the ERK cascade in vascular smooth muscle cells (Takahashi and Berk, 1998; Zou et al., 1998), no definitive evidence exists for activation of ERK by transmural pressure per se in vascular smooth muscle cells. Two other MAPK cascades, JNK and p38 MAPK, have been characterized in mammalian cells; the former is activated by cellular stress including cell stretch and shear stress while the latter is activated in response to inflammatory cytokines, endotoxins, and osmotic stress (Force and Bonventre, 1998). Nonetheless, it remains to be determined whether these kinases are involved in the pressure-induced DNA synthesis.

We have recently developed a computer-operated pressurizing system that allows investigation of the effect of pure pressure on cell functions without shear stress or stretch (Kato et al., 1999). In the present study, we used our system to examine the potential roles of several protein kinases in pressure-induced cell proliferation in cultured rat vascular smooth muscle cells.

2. Methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), 0.05% trypsin–0.02% EDTA and antibiotics–antimycotic mixture (penicillin 10,000 U/ml, streptomycin 10,000 μg/ml amphotericin B 25 μg/ml) were obtained from Gibco (Grand Island, NY). Bovine serum albumin was from Intergen (Purchase, NY) and fetal bovine serum was from Cansera International (Rexdale, Ontario, Canada). Genistein, $GdCl_3$, elastase, leupeptin and orthovanadate were from Sigma (St. Louis, MO). PD98059 (2′-amino-3′-methoxyflavone), SB203580 (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole) and bisindolylmaleimide I were from Calbiochem-Novabiochem (La Jolla, CA). Lisinopril was a kind gift from Zeneca (London, UK) and losartan was a gift from Merck (Whitehouse Station, NJ). Polyclonal antibodies against Tyr204-phosphorylated p44/42 MAPK, Tyr185-phosphorylated JNK and Tyr182-phosphorylated p38 MAPK, and Phototope®-HRP Western blot detection kit were from New England Biolabs (Beverly, MA). Collagenase was from Worthington Biochemical (Freehold, NJ). [^{3}H]thymidine and [γ-^{32}P]ATP were from Amersham Japan (Tokyo, Japan). Cellulose acetate filters (0.45 mm thick) were from Whatman (Goettingen, Germany). All other chemicals were of analytical grade and from Nacalai Tesque (Kyoto, Japan).

2.2. Cell culture

Dispersed vascular smooth muscle cells were prepared by enzyme digestion of aorta from male Sprague–Dawley rats (250–300 g) using the procedure described by Okazaki et al. (1994). The harvested cells were cultured in DMEM containing 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin B (0.25 μg/ml), 2 mM L-glutamine, and 10 mM HEPES (pH 7.4). Cultured cells were then incubated at 37 °C in humidified atmosphere of 95% air–5% CO_2. The medium was changed every 2 or 3 days and passages 3–5 were used throughout experiments. The cells were then incubated in a defined serum-free medium for 2 days before each experiment.

2.3. Pressure loading apparatus

We used a pressure loading apparatus developed in our laboratory described previously by Kato et al. (1999). Briefly, the apparatus consists of a resealable steel chamber with inlet and outlet ports (Miwa, Osaka, Japan) and the inlet port was connected through a tube to a reservoir of compressed helium while the exit port was connected through a tube to a sphygmomanometer and a computer-operated air-release valve. The culture plates used for assays of DNA synthesis (24-well plate) and cell proliferation (96-well plate) were placed on a warm plate (37 °C) inside the chamber. The partial pressure of O_2, CO_2, temperature and pH in the incubation medium in the plates remained constant throughout the experiments (data not shown). Cell viability was assessed with light microscopy, and was always >90% throughout experiments. Cells were morphologically intact, and the number of detached cells was negligible during experiments.

2.4. DNA synthesis

DNA synthesis was assessed by incorporation of [^{3}H]thymidine into cells as previously described by Kato et al.

(1999). Briefly, vascular smooth muscle cells were seeded at a density of 5×10^4 cells/well in a 24-well plate, rendered quiescent and subjected to a pressure loading from 40 to 160 mm Hg for 5 to 60 min. The cells were incubated in an incubator at 37 °C in humidified atmosphere of 95% air–5% CO_2. Twenty-two hours after pressurization, 1 μCi/ml [^{3}H]thymidine was added to each well and the cells were further incubated for an additional 2 h. Cells were then washed twice with ice-cold phosphate-buffered saline, once with 5% (w/v) trichloroacetic acid, ethylalcohol/diethylether (3:1, v/v), and then harvested with 0.3 M NaOH. After neutralization with 0.6 M HCl, the suspension was passed through a cellulose acetate filter, and the retained radioactivity was determined with a liquid scintillation spectrometer (LS7000; Beckman, Fullerton, CA).

2.5. Cell proliferation

Cells (8×10^3/well) were seeded onto 96-well plates, and rendered quiescent. Cells were pressurized at 120 mm Hg for 30 min, and incubated in an incubator at 37 °C in humidified atmosphere of 95% air–5% CO_2. Forty-eight hours after pressurization, cell numbers were determined with the Abacus Cell Proliferation Kit (Clontech, Palo Alto, CA). Briefly, cells were assayed for acid phosphatase activity using the instructions provided by the manufacturer (Ryden and Ibanez, 1996; Trupp et al., 1996). Incubation with the substrate solution was performed for 90 to 120 min at 37 °C. Absorbance was measured at 405 nm with an automatic plate reader (Bio-Rad Laboratories, Hercules, CA).

2.6. Effect of conditioned medium on DNA synthesis

Conditioned medium from pressurized cells was added to non-pressurized cells at atmospheric pressure and incubated at 37 °C in humidified atmosphere of 95% air–5% CO_2. After incubation for 22 h, 1 μCi/ml [^{3}H]thymidine was added to each well and cells were further incubated for another 2 h. In some experiments, incorporation of radioactivity into DNA was measured as described above.

2.7. Effects of inhibitors for protein kinase C, tyrosine kinase, angiotensin II receptor, angiotensin converting enzyme, and $GdCl_3$ on pressure-induced DNA synthesis

Cultured cells in 24-well plates were pretreated with inhibitors for protein kinase C (bisindolylmaleimide I), tyrosine kinase (genistein), a specific extracellular signal-regulated kinase kinase (MEK) inhibitor (PD98059) and a specific p38 MAPK inhibitor (SB203580) for 30 min before and during pressurization. $GdCl_3$ (10 μM), an inhibitor for stretch-activated mechanosensitive channels, was also added for 30 min before and during pressurization, which is known to inhibit the channel activity. To determine the involvement of the renin–angiotensin system in pressure-induced DNA synthesis, cells in 24-well plates were pretreated with 10 μM lisinopril, an inhibitor for angiotensin converting enzyme, and 1 μM losartan, an angiotensin receptor type 1 antagonist, for 30 min before and during the experiment.

2.8. Assay of ERK activity

Quiescent cells (5×10^4/well) grown on 24-well plates were stimulated by the applied pressure and the reaction was terminated by harvesting the cells with ice-cold lysis buffer (10 mM Tris–HCl (pH 7.4), 20 mM NaCl, 2 mM

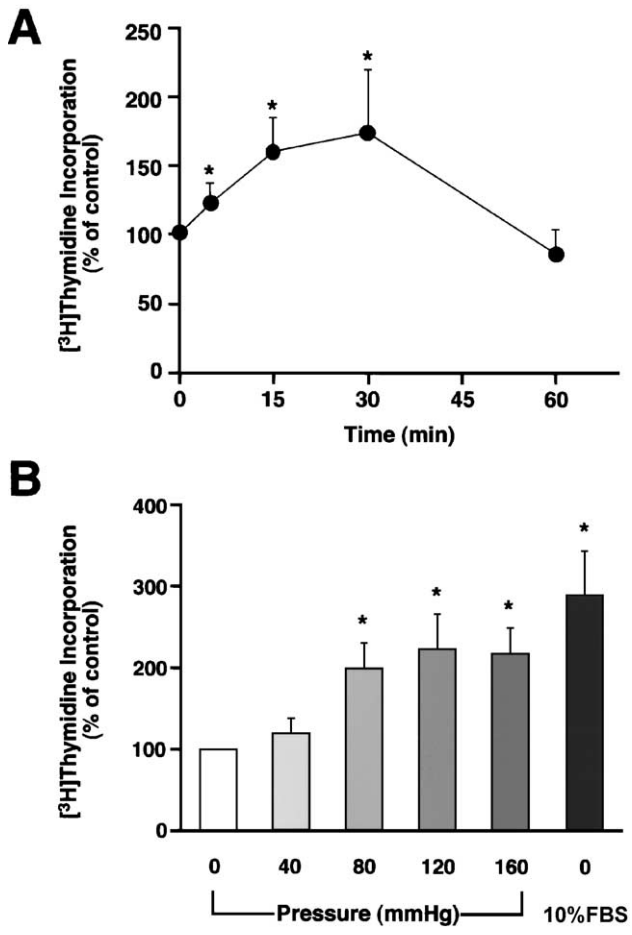

Fig. 1. Effects of duration (A) and intensity (B) of externally applied pressure on [^{3}H]thymidine incorporation in rat vascular smooth muscle cells. (A) Vascular smooth muscle cells in serum-free DMEM were subjected to a pressure of 120 mm Hg for the indicated periods. Twenty-two hours after pressure loading, the cells were further incubated for 2 h with [^{3}H]thymidine, and DNA synthesis was determined. Data are mean ± S.D. of four experiments. * $P < 0.05$ compared with control value (0 mm Hg). (B) Vascular smooth muscle cells in serum-free DMEM were subjected to a high pressure for 30 min at the indicated pressure values. In case of 10% fetal bovine serum (FBS), vascular smooth muscle cells were incubated in DMEM containing 10% fetal bovine serum. Twenty-two hours after pressure loading or stimulation with fetal bovine serum, the cells were further incubated for 2 h with [^{3}H]thymidine, and DNA synthesis was determined. Data are mean ± S.D. of five experiments. * $P < 0.05$ compared with control value (0 mm Hg).

EGTA, 1 mM orthovanadate, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride with 10 μg/ml leupeptin, and 10 μg/ml aprotinin). The samples were centrifuged at 14,000 × *g* for 5 min at 4 °C, and the supernatant was assayed for ERK activity by measuring the incorporation of [γ-^{32}P]ATP into KRELVEPLTPAGEAPNQALLR, a synthetic peptide, as a specific ERK substrate using an enzyme assay kit (Amersham). The reaction was carried out with the cell lysate (1 μg protein) in 75 mM HEPES buffer (pH 7.4), containing 1.2 mM $MgCl_2$, 2 mM substrate peptide, and 1.2 mM ATP, 1 μCi of [γ-^{32}P]ATP for 30 min at 30 °C. The resultant solution was applied to a phosphocellulose membrane and extensively washed in 1% acetic acid and then in distilled water. Radioactivity trapped on the membrane was measured by liquid scintillation counter (LS7000; Beckman).

2.9. Assay of MAPK phosphorylation

Quiescent cells (1×10^5/well) grown on a 12-well plate were pressurized in serum-free medium and the reaction was terminated by sonicating the cells with 50 μl sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) buffer, pH 6.8, containing 62.5 mM Tris–HCl, 2% SDS, 10% glycerol, 50 mM dithiothreitol, and 0.1% w/v bromophenol blue. Samples were boiled for 5 min and centrifuged at 10,000 × *g* for 5 min, and the supernatants were subjected to 10% SDS-PAGE. Proteins in the gel were transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA) by electroblotting. The membrane was treated with each primary antibody against ERK, JNK or p38 MAPK. After incubation with an appropriate secondary antibody, immunoreactive proteins were detected using the Phototope®-HRP Western Detection Kit. The level of MAPK phosphorylation was determined from the immunoblots by densitometric analysis and was corrected for amounts of MAPK protein on the membrane.

2.10. Transfection of antisense oligonucleotides for ERK into cells

Cells at 60–70% confluence were treated with an ERK antisense (5′-GCCGCCGCCGCCGCCAT-3′), or scrambled nucleotide (control) (5′-CGCGCGCTCGCGCACCC-3′) which were phosphorothioate-modified (BIOMOL, Plymouth Meeting, PA). The oligonucleotides were prepared in serum-free DMEM (final concentration, 5 μM) and then incubated for 15 min at room temperature with a transfection reagent in the TransFast™ Transfection Kit (Promega) at a ratio of 1:1 (w/v). Cells were washed twice with serum-free DMEM and oligonucleotide/transfection reagent mixture was added to each well, and the cells were incubated for 1 h at 37 °C in 5% CO_2. After incubation, cells were gently overlaid with DMEM containing 10% fetal bovine serum. After 24 h, cells were rendered quiescent and subjected to a pressure of 120 mm Hg for 30 min. DNA synthesis and immunoblotting were performed as described above.

2.11. Statistical analysis

Data are expressed as mean ± S.D. Differences between groups were examined for statistical significance using

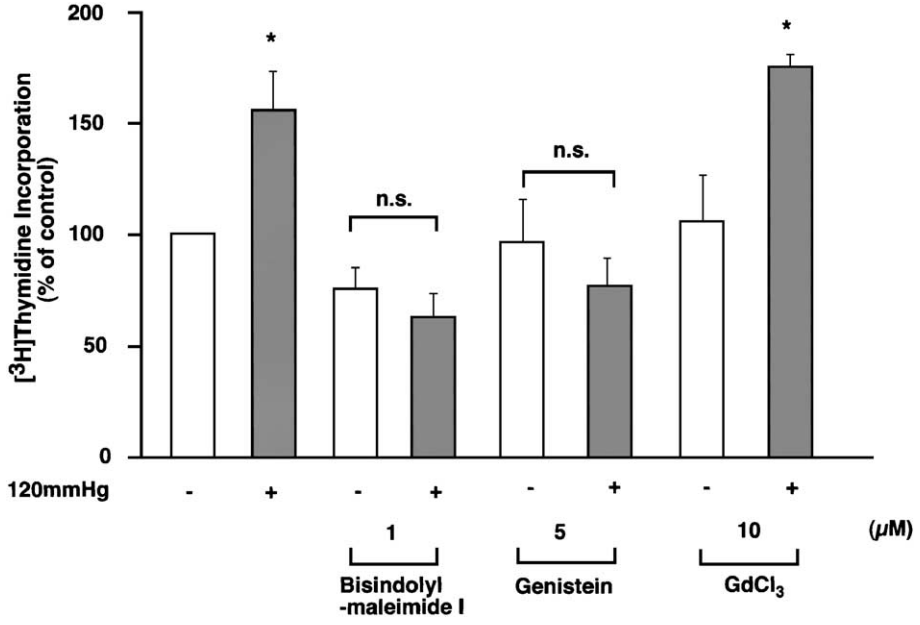

Fig. 2. Effects of inhibitors for protein kinase C and tyrosine kinase or $GdCl_3$ on pressure-induced [^{3}H]thymidine incorporation. Vascular smooth muscle cells were subjected to a pressure of 120 mm Hg for 30 min in the absence or presence of bisindolylmaleimide I (1 μM), genistein (5 μM) or $GdCl_3$ (10 μM), and DNA synthesis was determined. Data are mean ± S.D. of four experiments. * $P < 0.05$ compared with 0 mm Hg, no inhibitor.

analysis of variance (ANOVA) followed by Fisher's test. A P value of <0.05 denoted the presence of a statistically significant difference.

3. Results

3.1. Effects of applied pressure on vascular smooth muscle cell proliferation

Exposure of cultured rat vascular smooth muscle cells to 120 mm Hg pressure for 5 to 30 min significantly enhanced [^{3}H]thymidine incorporation in a time-dependent manner (Fig. 1A). Exposure to atmospheric pressure (0 mm Hg) for periods up to 60 min did not significantly change [^{3}H]thymidine incorporation. Exposure of cells to 120 mm Hg pressure for 60 min tended to decrease DNA synthesis rate compared to pressurization for 30 min (Fig. 1A). We also examined the relationship between applied pressure and DNA synthesis. Pressures of 80 to 160 mm Hg promoted [^{3}H]thymidine incorporation in a pressure-dependent manner (Fig. 1B), whereas pressure at 40 mm Hg had almost no effect. In our preliminary data, pressure of 200 mm Hg produced less [^{3}H]thymidine incorporation than pressure of 120 mm Hg and 160 mm Hg (data not shown). Treatment with 10% fetal bovine serum for 24 h increased [^{3}H]thymidine incorporation to $289 \pm 53\%$ ($n=4$, $P<0.05$) of the control level (Fig. 1B). Based on the above findings, vascular smooth muscle cells were exposed to 120 mm Hg pressure for 30 min. Under such conditions, the cell number increased significantly to $110 \pm 7.8\%$ ($n=5$, $P<0.05$) of the control level. These conditions were used in the remaining experiments.

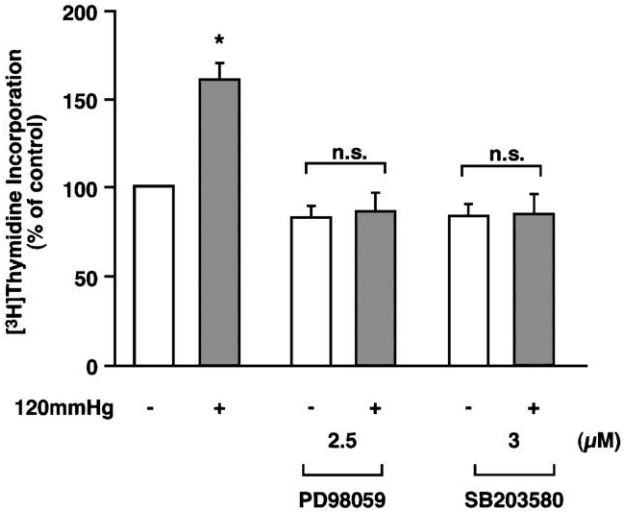

Fig. 3. Effects of PD98059 or SB203580 on pressure-induced [^{3}H]thymidine incorporation. Vascular smooth muscle cells were subjected to a pressure of 120 mm Hg for 30 min in the absence or presence of PD98059 (2.5 μM) or SB203580 (3 μM), and DNA synthesis was determined. Data are mean ± S.D. of three experiments. * $P<0.05$ compared with 0 mm Hg, no inhibitor.

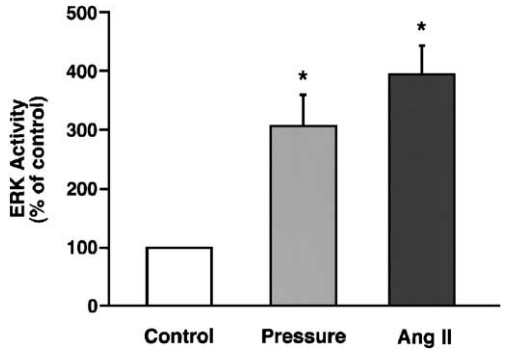

Fig. 4. Effects of applied pressure on ERK activity. Vascular smooth muscle cells cultured with serum-free DMEM for 48 h were incubated at atmospheric pressure or 120 mm Hg for 2 min, and ERK activity was determined with an ERK enzyme assay kit. Angiotensin II (Ang II) (10 μM) was used as a positive control of ERK activation. Vascular smooth muscle cell cultures were incubated with angiotensin II for 10 min, and ERK activity was determined. Data are mean ± S.D. of three experiments. * $P<0.05$ compared with control.

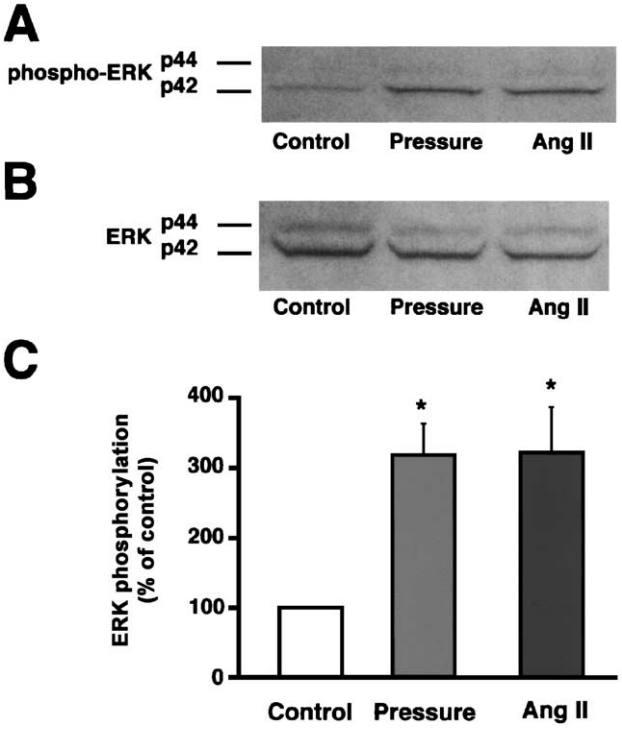

Fig. 5. Effects of applied pressure on phosphorylation of ERK. Cell extracts (20 μg of protein) were prepared from vascular smooth muscle cells exposed to pressure of 120 mm Hg for 2 min or incubated with angiotensin II (Ang II) (10 μM) for 10 min, and subjected to immunoblotting and immunoreactivity was detected (A) with an anti-phospho-ERK antibody or (B) an anti-ERK antibody. Angiotensin II was used as a positive control of phosphorylation of ERK. A representative immunoblot from three independent experiments is shown. (C) Levels of ERK phosphorylation were determined from the immunoblots by densitometric analysis (mean ± S.D., $n=3$). * $P<0.05$ compared with control.

3.2. Effects of inhibitors of protein kinase C and tyrosine kinase or $GdCl_3$ on pressure-induced DNA synthesis

Treatment of vascular smooth muscle cells with a specific protein kinase C inhibitor bisindolylmaleimide I (1 μM) significantly reduced pressure-induced DNA synthesis (Fig. 2). A specific tyrosine kinase inhibitor Genistein (5 μM) also inhibited pressure-induced DNA synthesis significantly (Fig. 2). The use of stretch-activated mechanosensitive channel inhibitor, $GdCl_3$, even at 10 μM, failed to inhibit pressure-induced DNA synthesis (Fig. 2).

3.3. Effects of PD98059 and SB203580 on pressure-induced DNA synthesis

PD98059, a specific MAPK kinase inhibitor, reduced pressure-induced DNA synthesis when used at 2.5 μM (Fig. 3). Furthermore, SB203580, a specific p38 MAPK inhibitor, also significantly inhibited pressure-induced DNA synthesis when used at 3 μM (Fig. 3).

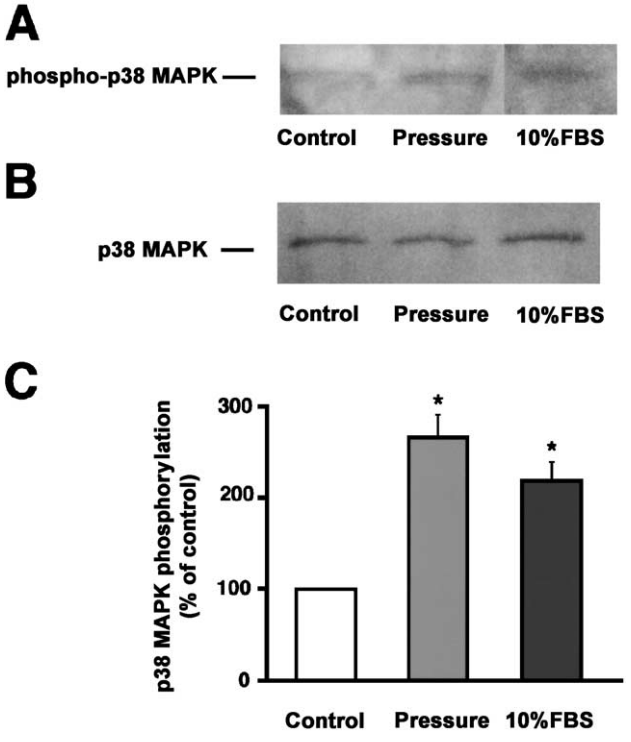

Fig. 6. Effects of applied pressure on phosphorylation of p38 MAPK. Cell extracts (20 μg of protein) were prepared from vascular smooth muscle cells exposed to pressure of 120 mm Hg for 10 min or stimulated with 10% fetal bovine serum (FBS) for 10 min, and subjected to immunoblotting with (A) an anti-phospho-p38 MAPK antibody or (B) an anti-p38 MAPK antibody. A representative immunoblot from three independent experiments is shown. (C) Levels of p38 MAPK phosphorylation were determined from the immunoblots by densitometric analysis (mean ± S.D., $n=3$). $^*P<0.05$ compared with control.

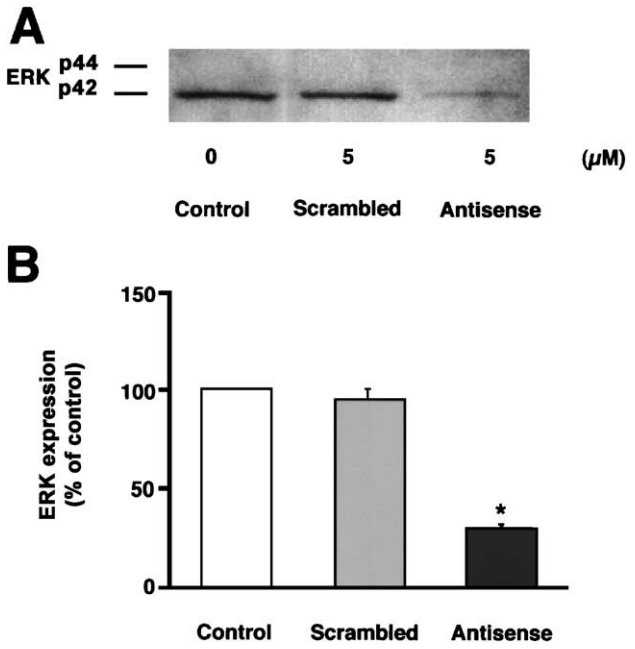

Fig. 7. Effects of pretreatment with ERK antisense oligonucleotide on ERK expression in vascular smooth muscle cells. (A) Cell extracts (20 μg of protein) were prepared from vascular smooth muscle cells transfected with antisense oligonucleotide against ERK or scrambled oligonucleotide, and subjected to immunoblotting with an anti-ERK antibody. Untransfected cells served as a control. The blot is a representative of at least three independent experiments. (B) The amount of ERK was determined from the immunoblots by densitometric analysis (mean ± S.D., $n=3$). $^*P<0.05$ compared with control.

3.4. Effect of applied pressure on ERK activity

Application of pressure at 120 mm Hg for 2 min increased ERK activity by 3.1-fold compared to the control (Fig. 4). ERK activity was significantly increased at 1 min after application of pressure at 120 mm Hg. The peak ERK activity was noted at 2 min after pressurization but the activity gradually returned to the basal level at 30 min (data not shown). Angiotensin-II (10 μM), a peptide known to

Table 1
Pretreatment of antisense oligonucleotide for ERK inhibits pressure-induced [^{3}H]thymidine incorporation in vascular smooth muscle cells

Treatment	[^{3}H]thymidine incorporation (cpm)
Scrambled	3353 ± 148
Pressure + scrambled	4148 ± 78[a]
Antisense	3506 ± 48
Pressure + antisense	3061 ± 302

Vascular smooth muscle cells were incubated with 5 μM scrambled oligonucleotide or antisense oligonucleotide for 1 h in DMEM containing liposomes (TransFast™ Transfection reagent), and were subjected to atmospheric pressure (0 mm Hg) or 120 mm Hg for 30 min. Twenty-two hours after pressure loading, cells were further incubated for 2 h with [^{3}H]thymidine, and DNA synthesis was determined. Data are mean ± S.D. of three experiments.

[a] $P<0.05$ compared with scrambled value (0 mm Hg).

increase ERK activity in vascular smooth muscle cells (Takahashi et al., 1997), also increased ERK activity by 3.9-fold from basal level at 10 min after stimulation (Fig. 4). ERK activity induced by angiotensin II was significantly increased at 2 min and reached a peak level at 10 min (data not shown).

3.5. Effect of applied pressure on phosphorylation of MAPK

Phosphorylation level of ERK was significantly increased after 2 min of pressurization compared to the control (pressure = 0) (Fig. 5A), while the amount of ERK proteins almost remained unchanged after pressurization (Fig. 5B). It is known that other MAPK, JNK and p38 MAPK, are activated in parallel with phosphorylation of ERK after stimulation by various stresses (Hamada et al., 1998). We found that the level of p38 MAPK phosphorylation was significantly increased at 10 min after pressurization (Fig. 6A). The amount of p38 MAPK protein in the cells remained unchanged during exposure to pressure (Fig. 6B). In contrast, JNK was not phosphorylated after pressurization for 10 min (data not shown).

3.6. Effect of an antisense oligonucleotide against ERK on pressure-induced DNA synthesis

ERK expression was suppressed by transfection of an antisense oligonucleotide against ERK at a concentration of 5 μM. In contrast, scrambled oligonucleotide had no effects on the expression of ERK (Fig. 7). As shown in Table 1, in ERK-deficient cells by transfection of an antisense oligonucleotide against ERK, pressure-induced increase in [^{3}H]thymidine incorporation was abolished. On the other hand, in the presence of the scrambled oligonucleotide, vascular smooth muscle cells maintained pressure-induced proliferative capacity (Table 1).

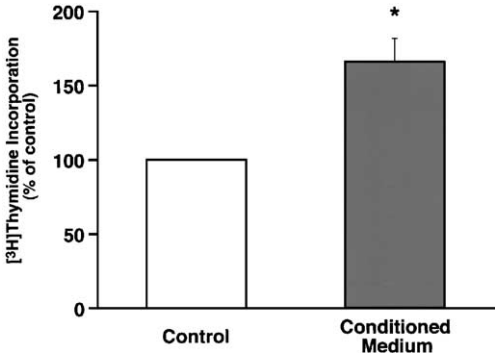

Fig. 8. Effects of conditioned medium with or without applied pressure on [^{3}H]thymidine incorporation. Vascular smooth muscle cells were incubated in culture medium from cells that had been exposed to atmospheric pressure (control) or pressure of 120 mm Hg for 30 min, and DNA synthesis was determined. Data are mean ± S.D. of three experiments. * $P < 0.05$ compared with values at 0 mm Hg for 30 min (control).

3.7. Effects of conditioned medium on vascular smooth muscle cell proliferation

To investigate the contribution of humoral factors that might be released from the cells in response to applied pressure, the culture medium of cells that had been subjected to 120 mm Hg pressure for 30 min was added to cultured vascular smooth muscle cells incubated at atmospheric pressure. The conditioned medium significantly activated DNA synthesis rate to 166 ± 15.6% compared to cells treated with the medium without pressure (Fig. 8). Angiotensin II is reported to be released from cardiac myocytes in response to stretch and causes hypertrophy of cardiac cells (Sadoshima et al., 1993; Yamazaki et al., 1996), while addition of angiotensin converting enzyme inhibitor lisinopril (10 μM) or angiotensin II type1 receptor antagonist losartan (1 μM) did not inhibit pressure-induced DNA synthesis in vascular smooth muscle cells (data not shown), suggesting that substances that affect renin–angiotensin system are not likely to be involved in the pressure-induced DNA synthesis.

4. Discussion

Stretch and shear stress activate several protein kinases and subsequently cause cell proliferation and vascular remodeling in vascular smooth muscle cells (Lehoux and Tedgui, 1998). However, the role of several protein kinases in transmural pressure-induced proliferation of vascular smooth muscle cells remains undetermined. In the present study, we investigated the role of several protein kinases in the pressure-induced DNA synthesis and cell proliferation in cultured rat vascular smooth muscle cells by using a system that eliminates the effects of stretch and shear stress (Kato et al., 1999). Our results demonstrated that ERK and/or p38 MAPK but not JNK cascades mediate the pressure-induced DNA synthesis in addition to the protein kinase C and the tyrosine kinase pathways.

Stretch has been reported to increase DNA synthesis and cell proliferation via the protein kinase C pathway in vascular smooth muscle cells and mesangial cells (Akai et al., 1994; Chaqour et al., 1999). Hishikawa et al. (1994) reported that transmural pressure induces activation of phospholipase C and protein kinase C, thereby promoting DNA synthesis in vascular smooth muscle cells. They also showed the involvement of the tyrosine kinase pathway in pressure-induced DNA synthesis based on the inhibitory effect of a tyrosine kinase inhibitor on pressure-induced DNA synthesis (Hishikawa et al., 1994). In the present study, we showed that not only inhibitors for protein kinase C but also those for tyrosine kinases inhibited the pressure-induced DNA synthesis in vascular smooth muscle cells. These results are consistent with our previous results in mesangial cells (Kato et al., 1999) and support the notion that protein kinase C and tyrosine kinase cascades play an

important role in pressure-induced DNA synthesis (Hishikawa et al., 1994; Kato et al., 1999).

In vertebrates, activation of tyrosine kinases or protein kinase C by extracellular stimuli that cause proliferative or hypertrophic effects converges to activate a set of cellular protein kinase cascades such as MAPK (Force and Bonventre, 1998). ERK are serine/threonine protein kinases that are activated by the upstream Ras–Raf-mitogen-activated protein kinase/MEK in response to several growth factors and mechanical forces (Brown et al., 1998; Force and Bonventre, 1998; Graf et al., 1997; Kusuhara et al., 1997; Lu et al., 1998; Ozaki et al., 1999; Takahashi and Berk, 1996; Takahashi et al., 1997; Tseng et al., 1995; Yamakawa et al., 1998). Many studies have demonstrated that the ERK cascade is critical to proliferation of many types of cells (Force and Bonventre, 1998). The fact that PD98059 inhibited the pressure-induced DNA synthesis in our study suggests that the ERK cascade is involved in the proliferative effects of transmural pressure. This is supported by our finding that pressure rapidly increased activation and phosphorylation of ERK. Kawata et al. (1998b) have also demonstrated that the ERK cascade plays an important role in pressure-induced cell proliferation in mesangial cells. Our experiments using cells that had been treated with a specific antisense oligonucleotide against ERK also demonstrated the requirement of ERK in pressure-induced DNA synthesis in vascular smooth muscle cells. Collectively, these results indicate that ERK activation is essential for pressure-induced DNA synthesis. Thus, modulation of ERK activity may be a key control step in the regulation of pathophysiological processes of blood vessels such as growth and vascular remodeling.

In contrast to ERK, JNK and p38 MAPK are activated by a number of cellular stresses including inflammatory cytokines (tumor necrosis factor-α and interleukin-1β), heat shock, osmotic stress, cell stretch and shear stress (Force and Bonventre, 1998). These pathways are reported to be involved in the transduction of growth inhibitory signals or apoptotic signals in certain cell types (Force and Bonventre, 1998). A number of cellular stresses activate both p38 MAPK and JNK (Force and Bonventre, 1998), however, our present study showed that pressure activated only p38 MAPK but not JNK. We also showed that SB203580 inhibited pressure-induced DNA synthesis, suggesting that the p38 MAPK cascade is also involved in pressure-induced DNA synthesis. The mechanism by which the p38 MAPK pathway mediates the pressure-induced DNA synthesis remains to be elucidated. Our results that JNK may not be involved in pressure-induced DNA synthesis are consistent with the recent report by Kawata et al. (1998b) using mesangial cells. However, Ozaki et al. (1999) reported opposite results about the effect of applied pressure on activation of JNK and p38 MAPK using human aortic vascular smooth muscle cells, which suggest that the mechanisms that activated the JNK and p38 MAPK pathways may be different between human and rat vascular smooth muscle cells. On the other hand, studies from other laboratories showed that shear stress and stretch could promote JNK activity in cardiac myocytes and vascular smooth muscle cells (Hamada et al., 1998; Komuro et al., 1996). Combined together, these results suggest that several pathways of MAPK activation exist depending on the application of different physical stresses among transmural pressure, shear stress and stretch.

Stretch-activated ion channels or mechanosensitive ion channels are considered candidate structures that transduce signals from mechanical forces to the cell membrane (Hajduczok et al., 1994). However, it is unlikely that stretch-activated ion channels are involved in pressure-induced DNA synthesis in our study, because the stretch-activated mechanosensitive channel blocker $GdCl_3$ did not have any effects on the pressure-induced DNA synthesis of vascular smooth muscle cells.

In aortic endothelial cells, shear stress by itself causes the release of some cytokines such as interleukin-1 and interleukin-6 (Sterpetti et al., 1993), while transmural compression enhances the release of endothelin-1 through activation of phospholipase C and protein kinase C (Hishikawa et al., 1995). Released cytokines or active peptides are reported to cause physical cellular responses such as cell proliferation or hypertrophy (Sadoshima et al., 1993; Yamazaki et al., 1996). In the present study, conditioned medium prepared from the pressurized vascular smooth muscle cells also promoted DNA synthesis, suggesting that substances that promote cell proliferation could be released from the pressurized vascular smooth muscle cells. The present results are different from those reported by Kawata et al. (1998a) and Hishikawa et al. (1994), who demonstrated the lack of any proliferative substances produced by the cells exposed to pressure in their experimental conditions. Angiotensin II has been shown to play an important role in the pathogenesis of many cardiovascular diseases, including hypertension and atherosclerosis (Berk and Corson, 1997; Rosendorff, 1998). Apart from its vasoconstrictor property, angiotensin II is a potent hypertrophic growth factor for vascular smooth muscle cells (Berk et al., 1989; Geisterfer et al., 1988; Holycross et al., 1993). In cardiac myocytes, angiotensin II is released by stretch and it subsequently activates myocyte hypertrophy in an autocrine manner (Sadoshima et al., 1993; Yamazaki et al., 1996). In our hands, since the angiotensin converting enzyme inhibitor lisinopril and angiotensin II type1 antagonist losartan could not suppress the pressure-induced cell proliferation, factors that promote cell proliferation other than angiotensin II are probably released from the pressurized vascular smooth muscle cells, thereby promoting DNA synthesis.

In conclusion, we have demonstrated that activation of ERK is essential for pressure-induced proliferation of vascular smooth muscle cells, in addition to activation of protein kinase C and tyrosine kinase pathways. We also showed that p38 MAPK but not JNK is involved in pressure-mediated events. Cell proliferation caused by these

multi-step processes of protein kinase activation by transmural pressure could be involved in the pathogenesis of atherosclerosis in hypertension. Understanding the mechanism by which high pressure promotes cell proliferation should be helpful in developing novel therapeutic strategies for the treatment of cardiovascular diseases caused by hypertension.

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