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Threshold-Dependent DNA Synthesis by Pure Pressure in Human Aortic Smooth Muscle Cells: Giα-Dependent and -Independent Pathways

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Mechanical forces related to pressure and flow are important for the etiology of atherosclerosis and hypertension. We hypothesized the presence of mechanosensors that were solely sensitive to pure atmospheric pressure in the absence of shear and tensile stresses. A pressure-loading apparatus was set up to examine the effects of atmospheric pressure on human aortic smooth muscle cells (HASMC). Pressure application of 140 to 180 mmHg produced DNA synthesis in a pressure-dependent manner. In contrast, pressure of 120 mmHg or less produced no significant change. Both extracellular signal-regulated kinase and c-Jun N-terminal kinase activities, but not p38 activity, were stimulated by pressures of more than 160 mmHg. Pertussis toxin (PTx) completely inhibited the pressure-induced increase of DNA synthesis under the high pressure of 200 mmHg. These data suggest that HASMC have a mechanosensing cellular switch for DNA synthesis which is sensitive to pure atmospheric pressure, and that the molecular switch is activated by pressure of more than 140 mmHg. The activation mechanism consists of PTx-sensitive and -insensitive pathways, and the former is activated by high pure pressure.

Abnormal growth and proliferation of vascular smooth muscle cells have been implicated in the pathogenesis of atherosclerosis and hypertension (1). Mechanical stresses are likely to be involved in this process, since it has been demonstrated that they regulate cell growth in many tissues (2). In arteries, for example, a mechanical stress related to pressure and flow is crucial in promoting blood vessel wall remodeling. Such vascular remodeling may have important clinical implications for the evolution of several vascular diseases, may alter vascular compliance in hypertension and atherosclerosis, and may cause vascular fragility and compensatory changes in atherosclerosis (3).

Various stresses have been studied, including cytokines, mitogens, ultraviolet light, oxidant stress, hyperosmolarity, heat stress and mechanical stress (4). The vascular endothelial and vascular smooth muscle cells covering the inner surface of blood vessels are constantly exposed to such stresses. The hemodynamic forces affecting endothelial cells include shear stress due to the frictional force of blood flow, and circumferential and pure pressure stresses due to transmural pressure. Recently, a number of studies have shown that shear stress to the vessel wall, which is one of the mechanical stresses generated by blood flow, modulates endothelial morphology and function (5). However, in an *in vitro* cell culture model, it has been shown that intracellular signalings produced by tensile and shear stresses may be influenced not only by pressure stress, but also by morphological and cytoskeletal changes of cells (6). Vascular smooth muscle cells make up the outer layer of the endothelium in blood vessels and are thus indirectly exposed to blood flow. Therefore, mechanosensing intracellular signal transductions in vascular smooth muscle cells are defined as a cellular response to transmural pure pressure and tensile stress in the absence of shear stress. In previous studies, tensile stress was reported to accelerate DNA synthesis and activate stretch-activated cation channels in vascular smooth muscle cells (7, 8). However, the molecular identities of these candidates for mechanosensitive receptors are currently unknown.

In this study, we hypothesized the presence of mechanosensors and intracellular signal transductions act-

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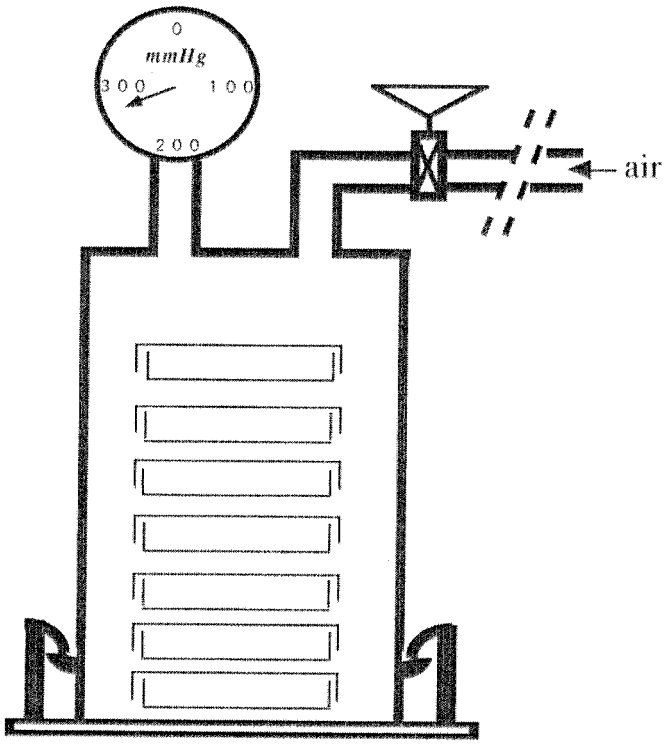

FIG. 1. Diagrammatic representation of the apparatus used in this study.

ing under pure atmospheric pressure in the absence of tensile and shear stresses. Based on this hypothesis, we evaluated the DNA synthesis and intracellular signalings of human aortic smooth muscle cells using an original pressure-loading apparatus that was capable of directly applying various levels of pure pressure (maximum, 300 mmHg) on HASMC, in order to examine the effect of pure pressure stress on mechanosensors without inducing morphological change of cells. In this manner, we determined the threshold of pressure for activating the mechanosensing cellular switch and clarified the differential intracellular signaling mechanisms involving PTx-sensitive and -insensitive heterotrimeric G-proteins.

MATERIALS AND METHODS

Cell culture. HASMC (Clonetics) were cultured in smooth muscle cell basal medium (SmBM; Clonetics) which was modified MCDB 131 containing 5% fetal bovine serum, gentamicin (50 μg/ml), amphotericin (50 μg/ml) and several growth factors: human epidermal growth factor (10 ng/ml), human fibroblast growth factor (2.0 ng/ml), and insulin (5.0 μg/ml). The cells were incubated at 37°C in a humidified, 5% CO_2 atmosphere. The 4th through 8th passages of HASMC were plated on 6-well plates for further investigation.

Pure pressure-loading apparatus. An original pure pressure-loading apparatus was designed to expose HASMC under pure atmospheric pressure stress (Fig. 1). The chamber allows for pumping in air or nitrogen gas to raise the internal pressure (maximum at 300 mmHg), and can be sealed tightly by placing several clamps at the bottom edge. Internal pressure levels were monitored with an aneroid barometer during the experiments. The compression chamber was established in the incubator and kept at 37°C, and a digital thermometer was mounted in the incubator to monitor the exact internal temperature. In the following series of experiments, the chamber was kept at 37°C, and the partial pressures of pO_2 and pCO_2 were theoretically preserved as constants according to the Boyle-Gay-Lussac's law. The potential of hydrogen (pH) in the culture medium was supported at a constant level (7.41 $\pm$ 0.02) during the experiments. It was not possible to monitor the actual morphological changes of pressurized cells in our system set-up. However, light microscopic investigations failed to find any changes in cell size or morphology during and after pressurization.

HASMC were cultured on 6-well plates for 48 hours (hrs). In 3 wells of each plate, the medium was changed to a starving medium (Dulbecco's modified Eagle's medium without serum). In the other 3 wells, the medium was changed to the same starving medium but containing PTx (0.1 μg/ml; Seikagaku, Japan). After 8 hrs of incubation, the plates were placed in the pure pressure-loading apparatus in the medium containing 10 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid, pH 7.4), exposed to various levels of atmospheric pressure (0 through 240 mmHg), and then incubated in an incubator for 1 or 3 hrs at 37°C.

DNA synthesis. [^{3}H]-Thymidine (TdR) incorporation into DNA was studied as a marker for DNA synthesis acceleration by pure atmospheric pressure. [^{3}H]-TdR (2 μCi/ml; Amersham) was added to the medium in all 6 wells of a 6-well plate. After pressure-loading procedures, the plate was incubated in a 37°C CO_2-incubator under normal pressure for 4 hrs. The cells were rinsed two times in ice-cold phosphate-buffered saline (PBS) followed by precipitation three times with ice-cold 10% trichloroacetic acid, and were lysed in 0.5N NaOH at 37°C by shaking for 30 min. The incorporation of [^{3}H]-TdR into DNA was quantified by pipetting the DNA hydrolysate into counting vials containing 4 ml of liquid scintillation cocktail (Ready Gel; Beckman). The protein concentration of HASMC was normalized by Lowry's method with bovine serum albumin as a standard (9). The counting results were expressed as disintegrations per protein and were expressed as a percent-increase compared to controls at the basal level (0 mmHg) for 1 and 3 hrs. All the experiments for each pressure were performed in triplicate, and were repeated 2 to 3 times.

Immunoblotting. HASMC were incubated on 100-mm dishes until 80% confluent. The medium was replaced with a starving medium. After 8 hrs of incubation, the dishes were placed in the pure pressure-loading apparatus in the medium containing 10 mM HEPES, and exposed to various levels of atmospheric pressure (0 mmHg, 120 through 180 mmHg, and 240 mmHg) for 3 hrs at 37°C. The cells were washed twice with ice-cold PBS, and harvested in a buffer containing 25 mM Tris-HCl (pH 6.8), 1% Triton X-100, 150 mM sodium chloride and protease inhibitors: benzamidine (100 μM), leupeptin (2 μM), aprotinin (0.15 μM), pepstatin A (1.5 μM), and phenylmethylsulfonyl fluoride (100 μM). The protein content of each sample was measured by Lowry's method. Samples (15 μg) were analyzed by SDS (sodium dodecyl sulfate)-polyacrylamide gel electrophoresis in a 12% gel using the Mini Gel Electrophoresis System (Marysol). Proteins were transferred to a nitrocellulose membrane (Hybond ECL; Amersham) by a Western Blotting apparatus (Semi-dry type; Marysol) with a buffer containing 20% methanol, 48 mM Tris-base, 78 mM glycine, and 0.0375% SDS for 2 hrs. The membranes were submerged for 1 hour in 4% non fat dry milk in TTBS (0.05% Tween-20, Tris-buffered saline, pH 7.4), followed by an incubation for 2 hrs in TTBS containing the appropriate primary antibodies (anti-active extracellular signal-regulated kinase (ERK), -active c-Jun N-terminal kinase (JNK), and -active p38 anti-rabbit polyclonal antibodies; Promega) in concentrations recommended by the manufacturer. The membranes were incubated in TTBS containing horseradish peroxidase-labeled donkey anti-rabbit Ig's (1:2500; Amersham), followed by washing three times in TTBS. The detection

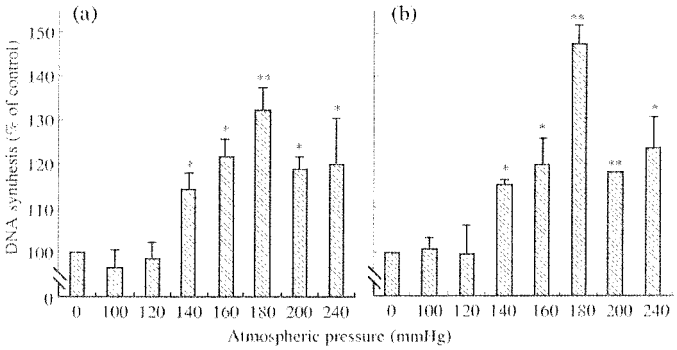

FIG. 2. Effect of various pure pressures for 1 hour (a) or 3 hours (b) on an increase of [^{3}H]-TdR incorporation. HASMC were pressurized under various pressures of 0, 100 to 200 (in 20-mmHg increments) and 240 mmHg, for 1 or 3 hours. [^{3}H]-TdR incorporation was determined as a marker of DNA synthesis, and was expressed as a percent-increase compared to controls. (a) HASMC were exposed to various pressures for 1 hour. Pure pressure-induced DNA synthesis was activated at atmospheric pressure of more than 140 mmHg. (b) HASMC were pressurized under various pressures for 3 hours. Pure pressure-induced DNA synthesis was activated by an atmospheric pressure of more than 140 mmHg, and DNA synthesis was induced in a pressure-dependent manner from 140 to 180 mmHg. Values are means ± SEM (n = 6–9). * $p < 0.05$ vs. controls; ** $p < 0.01$ vs. controls.

of the proteins was performed using a chemiluminescence method (ECL; Amersham). Active phosphorylated forms of ERK, JNK and p38 were scanned with a digital image analyzing system and quantified as NIH images.

Data analysis. All the results were expressed as means ± SEM, and statistical significance was assessed by Student's *t* test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Pure pressure-dependent acceleration of DNA synthesis in HASMC. With the pure pressure-loading apparatus, an atmospheric pressure of 240 mmHg was applied on cultured HASMC. DNA synthesis was measured as [^{3}H]-TdR incorporation into DNA. After one hour of pressure application, the pure pressure of 240 mmHg induced an approximately 20% increase in [^{3}H]-TdR incorporation compared to the control HASMC (Fig. 2). After more than 3 hrs, the degree of acceleration of DNA synthesis was similar.

Threshold of pure atmospheric pressure in acceleration of DNA synthesis. To determine the threshold of pure atmospheric pressure in acceleration of DNA synthesis, pure pressure was applied to HASMC for 1 or 3 hrs. Various levels of atmospheric pressure (0 mmHg, 100 through 200 mmHg, in 20-mmHg increments, and 240 mmHg) were applied to analyze the threshold of pure pressure in acceleration of DNA synthesis (Fig. 2). The atmospheric pressures of less than 120 mmHg produced no significant change in [^{3}H]-TdR incorporation at 1 and 3 hrs. However, pressure of 140 mmHg produced an approximately 20% increase in [^{3}H]-TdR incorporation ($p < 0.05$ vs. control) at 1 and 3 hrs. Pressures of more than 140 mmHg also produced an increase (14 ± 6% to 27 ± 4%; $p < 0.05$ vs. control). At 3 hrs, the degree of increase was dependent on pressure levels from 140 to 180 mmHg, with 180-mmHg pressure producing an increase of 49 ± 6% compared to control cells ($p < 0.01$ vs. control). 200-mmHg pressure significantly accelerated DNA synthesis at both 1 and 3 hrs. However, exposure to 200-mmHg pressure did not further increase the DNA synthesis.

Immunoblotting. Pure atmospheric pressure stimulated ERK1/2 and JNK activities. Pressure of more than 160 mmHg induced an activation of ERK1 (p44), and ERK1 was activated to a maximum level at pressure of 240 mmHg (increase in active form of ERK1 vs. control: 63.2 ± 12.1%). Pressures of more than 120 mmHg induced an activation of ERK2 (p42), and ERK2 was activated to a maximum level at pressure of 180 mmHg (increase in active form of ERK2 vs. control: 49.5 ± 7.3%) (Fig. 3a). JNK was activated at pressures of more than 160 mmHg in a pressure-dependent manner and to a maximum level at pressure of 240 mmHg (increase in active form of JNK vs. control: 45.7 ± 6.9%) (Fig. 3b). Application of pure atmospheric pressure induced no activation of p38 (Fig. 3c).

PTx effect on pure pressure-dependent DNA synthesis. Finally, we examined whether Gi-proteins are involved in pure pressure-dependent acceleration of DNA synthesis in HASMC. DNA-synthesis in acceleration under 200-mmHg pressure was completely inhibited by PTx (0.1 μg/ml) at 3 hrs (increase of DNA synthesis vs. control: 18.8 ± 2.7% at 1 hr and 18.0 ± 1.4% at 3 hrs in the absence of PTx; 4.4 ± 2.7% at 1 hr and 2.3 ± 2.9% at 3 hrs in the presence of PTx; Fig. 4b). In contrast, an acceleration of DNA synthesis by 160-mmHg pressure was not significantly inhibited at this time point (increase of DNA synthesis vs. control: 21.6 ± 2.5% at 1 hr and 27.4 ± 5.5% at 3 hrs in the absence of PTx; 16.5 ± 6.9% at 1 hr and 32.5 ± 5.5% at 3 hrs in the presence of PTx; Fig. 4a). PTx also failed to significantly inhibit the acceleration produced by 180-mmHg pressure (increase of DNA synthesis vs. control: 24.6 ± 4.3% at 1 hr and 40.1 ± 11.4% at 3 hrs in the absence of PTx; 24.2 ± 3.9% at 1 hr and 49.0 ± 6.0% at 3 hrs in the presence of PTx).

DISCUSSION

Mechanical forces are important modulators of cellular functions, and particularly in the cardiovascular system. Mechanical stress has various components, such as wall shear stress, tensile stress and pure pressure stress. In the case of shear stress, Ando *et al.* reported that a fluid shear stress of 1.3–4.1 dynes/cm^2 affected endothelial cell DNA synthesis during regeneration (10). Jo *et al.* reported that shear stress stim-

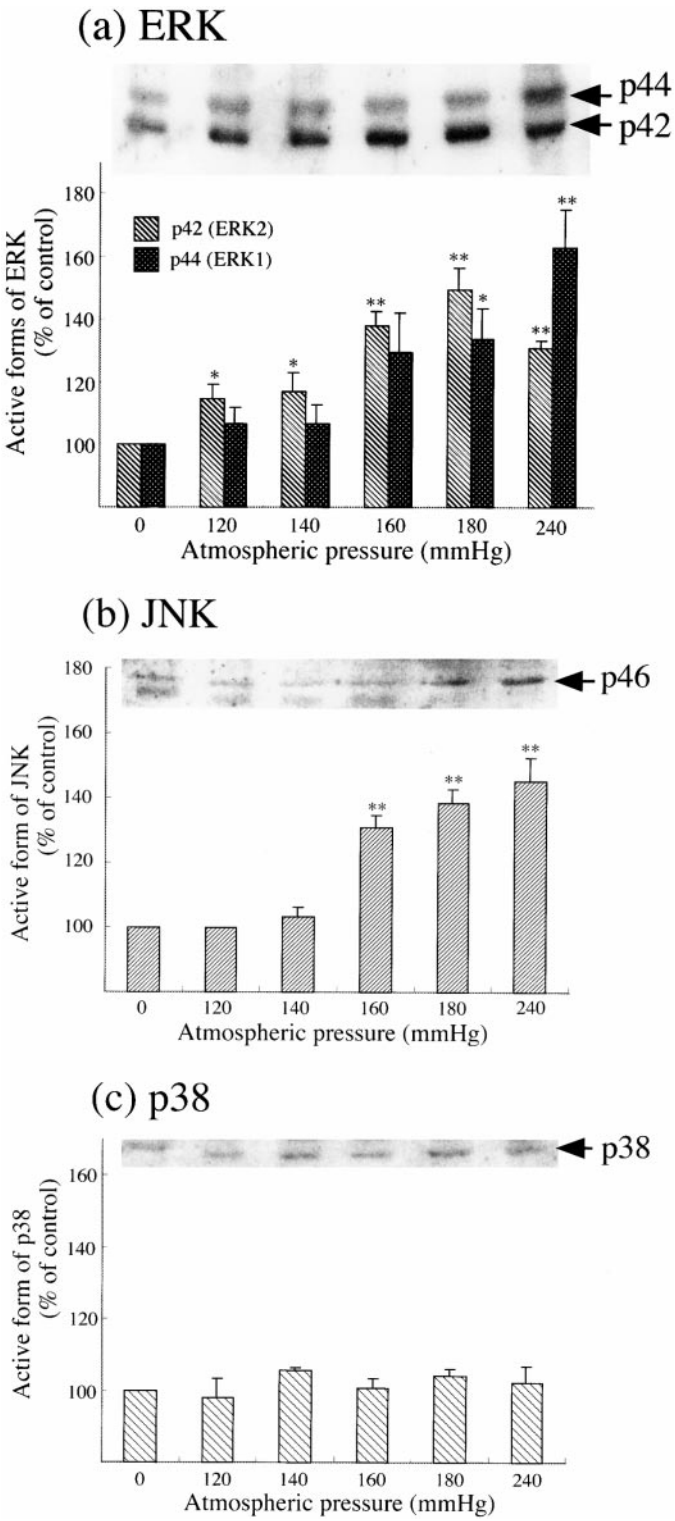

FIG. 3. Detection of active ERK (a), active JNK (b) and active p38 (c) in HASMC at various levels of pure atmospheric pressure. (a) ERK2 (p42) was activated at pressures of more than 120 mmHg, and to a maximum level at pressure of 180 mmHg. ERK1 (p44) was activated at pressures of more than 160 mmHg, and to a maximum level of pressure of 240 mmHg. (b) JNK was activated at pressure of more than 160 mmHg in a pressure-dependent manner. (c) p38 was not stimulated at pure atmospheric pressure. The bar graph shows densitometric analysis of active phosphorylated forms of ERK (a), JNK (b) and p38 (c). Values are means ± SEM (n = 3–5). * $p < 0.05$ vs. controls; ** $p < 0.01$ vs. controls.

ulated ERK and JNK activity in a force-dependent manner in endothelial cells (11). Shear stress is also known to activate phospholipase C and to generate inositol triphosphate (IP_3) and diacylglycerol. IP_3 releases Ca^{2+} from Ca^{2+} stores via IP_3 receptor, and diacylglycerol activates protein kinase C (PKC) (12, 13). Several PKC isozymes have been suggested to be involved in several downstream signalings, such as ERK activation, NF κ B-mediated gene transcription, *erg-1* transcription and activation of c-Src families (14–16). Recently, Cucina *et al.* reported that shear stress induced changes in the morphology and cytoskeletal organization of endothelial cells, and that these changes may be correlated to the functional change after exposure to shear stress (6). It is interesting to speculate that the focal adhesion complex plays an important role in shear stress-induced signalings, and that shear stress-induced signalings participate in intracellular cross-talk with integrin-coupled signal transductions (17, 18). In these studies, however, it is difficult to separate the direct effects of pure pressure from the indirect effects caused by the morphological change of cells. Hishikawa *et al.* reported that pure pressure stress promoted DNA synthesis in rat cultured vascular smooth muscle cells in an original pressure-loading apparatus, as determined by immunocytochemical assay (19). This study suggests the possible presence of a mechanosensing cellular switch that is solely sensitive to pure pressure stress. However, in experiments with such apparatus, the threshold pressure for determining the on and off status of the mechanosensing cellular switch has not been clarified.

In this study, we demonstrated that pure pressure stress accelerated an increase of DNA synthesis in the absence of shear stress and tensile stress, and determined the threshold of pure pressure stress which presumably activates the mechanosensing cellular switch. Using an original pressure-loading apparatus, we investigated various pressure levels from 0 to 240 mmHg. Low atmospheric pressure of less than 120 mmHg had no significant effect on DNA synthesis, while pressures of more than 140 mmHg induced an acceleration of DNA synthesis. From these results, we conclude that a mechanosensing cellular switch for DNA synthesis that was solely sensitive to pure pressure was "on" or "off" at over or under 140 mmHg, respectively. Pressures of 140 to 180 mmHg promoted an increase of [^{3}H]-TdR incorporation in a pressure-dependent manner at 1 and 3 hrs. Pressures of more than 200 mmHg also induced approximately 20% increase of DNA synthesis. However, the degree of accel-

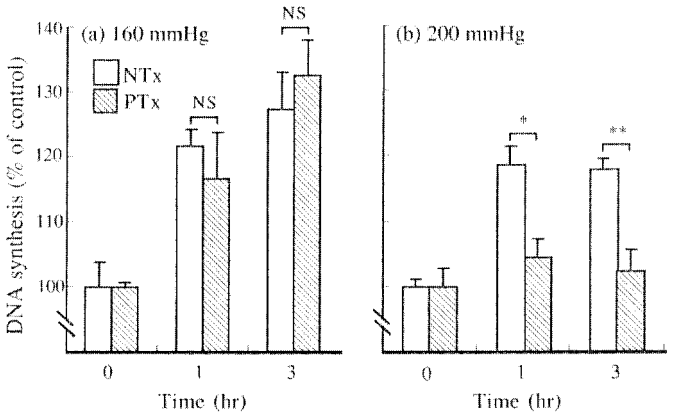

FIG. 4. Effect of PTx on a pure pressure-induced increase of [^{3}H]-TdR incorporation. HASMC were incubated for 8 hours in the absence (NTx) or presence of PTx. (a) PTx produced no significant effect on [^{3}H]-TdR incorporation into DNA synthesis under an atmospheric pressure of 160 mmHg. (b) PTx inhibited an increase in [^{3}H]-TdR incorporation especially under an atmospheric pressure of 200 mmHg. Values are means ± SEM (n = 6–9). NS, not statistically significant; * $p < 0.05$ and ** $p < 0.01$ vs. in the absence of PTx.

eration of DNA synthesis at pressures of more than 200 mmHg was similar and pressure-independent.

We also demonstrated the differential pathways in HASMC in response to pure atmospheric pressures. ERK1 was activated at pressures of more than 160 mmHg, and to a maximum level at pressure of 240 mmHg. ERK2 was activated at pressures of more than 120 mmHg, and to a maximum level at pressure of 180 mmHg. JNK was activated at pressures of more than 160 mmHg in a pressure-dependent manner. In contrast, p38 was not activated at pure atmospheric pressure. The mechanosensing mechanism stimulated by pure atmospheric pressure may consist of some differential pathways involving ERK and JNK signalings, and differential levels of pure pressure may activate each pathway. Moreover, differential activation of ERK and JNK in HASMC by pure atmospheric pressure may result in the selective phosphorylation and activation of transcription factors leading to selective gene regulatory events. It is not clear why the degrees of acceleration of DNA synthesis and activation of ERK2 were independent of pressure levels of more than 200 mmHg or why the pressure levels that accelerated DNA synthesis and activated ERK and JNK were different.

A role for heterotrimeric G-proteins in mechanical stress-induced signal transductions in endothelial cells has recently emerged (20, 21). It is known that both heterotrimeric G proteins and small G-proteins are activated by shear stress. Shear stress-induced activation of G-proteins results in several flow-initiated endothelial responses that regulate vascular tone and that release such vasodilators as nitric oxide and prostaglandin I_2, and such vasoconstrictors as endothelin (22–25). Therefore, we also investigated whether mechanosensing signaling pathways that are sensitive to pure pressure in HASMC were related to Gi-dependent pathways or Gi-independent pathways. At atmospheric pressures of 160 mmHg, PTx demonstrated no significant effect on [^{3}H]-TdR incorporation. However, at a high atmospheric pressure of 200 mmHg, PTx completely inhibited the increase of [^{3}H]-TdR incorporation. Pure pressure-induced DNA synthesis was produced through intracellular signaling pathways, including both Gi-dependent and -independent pathways. Although the molecular switch was "on" at an atmospheric pressure of more than 140 mmHg, an atmospheric pressure of below 180 mmHg may activate intracellular signalings predominantly through Gi-independent pathways, and a pressure of more than 200 mmHg may activate DNA synthesis predominantly through Gi-dependent pathways.

In summary, we demonstrated that HASMC had a mechanosensing molecular switch for DNA synthesis which was solely sensitive to pure atmospheric pressure, and the switch was "on" or "off" at over or under 140 mmHg, respectively. Moreover, mechanosensing cellular mechanisms may consist of some mechanosensors or intracellular pathways activated by different levels of pure atmospheric pressure. This study showed a change in dominance of Gi-dependent or Gi-independent intracellular signaling pathways in a pressure range from 160 to 200 mmHg. Recently, it has been reported that the minimum risk for cardiovascular mortality was reached at 138.8 mmHg for a mean systolic arterial pressure in a randomized clinical study of patients with hypertension (26). Activation of mechanosensing switches in HASMC at 140 mmHg may be involved in clinical cardiovascular events.

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