Static pressure drives proliferation of vascular smooth muscle cells via caveolin-1/ERK1/2 pathway

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Abstract

Intimal hyperplasia plays an important role in various types of vascular remodeling. Mechanical forces derived from blood flow are associated with the proliferation of vascular smooth muscle cells (VSMC). This contributes to many vascular disorders such as hypertension, atherosclerosis and restenosis after percutaneous transluminal angioplasty (PTA). In this study, we show that static pressure induces the proliferation of VSMC and activates its related signal pathway. VSMC from a rat aorta were treated with different pressures (0, 60, 90, 120, 150 and 180 mm Hg) in a custom-made pressure incubator for 24 h. The most active proliferation of VSMC was detected at a pressure of 120 mm Hg. Extracellular signal-regulated kinases 1/2 (ERK1/2) activation showed a peak at the pressure of 120 mm Hg at 4-h time point. Moreover, caveolin-1 expression was significantly inhibited by rising static pressure. Downregulation of VSMC proliferation could be found after PD98059 (ERK1/2 phosphorylation inhibitor) treatment. Our data also showed that a siRNA-mediated caveolin-1 knock down increased ERK1/2 phosphorylation and VSMC proliferation. These results demonstrate that static pressure promotes VSMC proliferation via the Caveolin-1/ERK1/2 pathway.

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Introduction

VSMC proliferation is involved in the common pathogenesis of hypertension, atherosclerosis and restenosis after percutaneous transluminal angioplasty (PTA) [1-4]. Mechanical force is an important factor in VSMC proliferation regulation [5]. Mechanical forces derived from blood flow include shear stress and vertical pressure. The effects of shear stress on endothelial cells and VSMC have been widely studied. It is clear that shear stress stimulates

Abbreviations: VSMC, vascular smooth muscle cells; ERK, extracellular signal-regulated kinase; CO2, carbon dioxide; DMEM, Dulbecco’s minimum eagle’s medium; MTT, 3-(4,5-dimethyl(2-thiazol-2-yl))-3,5-diphenyltetrazolium bromide; PKC, protein kinase C; EGFR, epidermal growth factor receptor

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nal cascade [13]. Caveolins associate with beta1 integrin, a sensor of mechanical force [14–18]. Focal adhesion kinase signaling regulates the expressions of caveolins and beta1 integrin [19]. Therefore, the caveolin-1/ERK1/2 pathway may play a role in mediating static pressure-induced VSMC proliferation.

In this study, we thus directly addressed these important issues, using our original pressure-adjustable cell incubator to examine the effects of static pressure on VSMC proliferation.

Materials and methods

Pressure incubator. The pressure-adjustable cell incubator is a custom-made, steel container with a door placed in a thermostat oven with a temperature controller. The humidified atmosphere in the container was 5% CO₂ and 95% air. The pressure in the container is controlled by a knob (pressure controller) between the container and the gas tank to adjust the mixed gas. The container links to a pH meter to monitor the pH of cellular media.

Cell culture. VSMC were isolated from rat thoracic aortas with a previously published technique [20,21]. VSMC were cultured with DMEM supplemented with 10% fetal bovine serum (GIBCO Life Technology, USA). VSMC between passage 3 and 12 were used. Cells were starved with serum-free DMEM for 24 h at subconfluence of 70%, USA). VSMC between passage 3 and 12 were used. Cells were subjected to serum-free starvation for another 24, and then treated with different pressures (0, 60, 90, 120, 150, 180 mm Hg) for different times. Thereafter, 50 μl of 1 mg/ml MTT in DMEM (without phenol red) was added into each well and incubated for 4 h at 37 °C. Formazan crystals were solubilized in 100 μl DMSO by shaking at room temperature for 10 min. Absorbance was measured at 570 nm using 630 nm as reference filter.

Trypan blue staining. VSMC were seeded at 5 × 10⁵ cells per well into 96-well plate and allowed to adhere for 24 h. Cells were subsequently subjected to serum-free starvation for another 24, and then treated with different pressures (0, 60, 90, 120, 150, 180 mm Hg) for different times. Thereafter, 50 μl of 1 mg/ml MTT in DMEM (without phenol red) was added into each well and incubated for 4 h at 37 °C. Formazan crystals were solubilized in 100 μl DMSO by shaking at room temperature for 10 min. Absorbance was measured at 570 nm using 630 nm as reference filter.

Flow cytometry. VSMC were trypsinized and washed twice with cold PBS and then resuspended in 50 μl cold PBS and 450 μl cold methanol and incubated for 1 h on ice. The cells were stained with 50 μg/ml propidium iodide (PI) (Sigma, USA) for 1 h, and the cell cycles were analyzed by a FACScan flow cytometer (EPICS-XL, Beckman Coulter, USA) at a wavelength of 488 nm. Western blot analysis. VSMC were washed with cold PBS three times and solubilized on ice with a lysis buffer (50 mM Tris–HCl, pH 8.0, 0.15 M NaCl, 1% Triton X-100, 0.1% SDS, 5 mg/ml sodium deoxycholate) in the presence of 10 μg/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM NaF and 1 mM Na3VO4 (Sigma, USA). An equivalent amount of protein from each sample was separated by SDS–PAGE concentration and measured with the bicinchoninic acid (BCA) assay (Hyclone–Pierce Company, USA), and then transferred to a polyvinylidene difluoride (PVDF) membrane for 100 V, 90 min with a Bio-Rad Mini-Protein II transfer apparatus (Bio–Rad Laboratories, USA). Blots were blocked overnight at 4 °C in Tris-buffered saline with 0.1% Tween-20 (TBS-T) and 5% nonfat dried milk (NFDM). Total caveolin-1, total ERK1/2 and p-ERK1/2 were detected with monoclonal antibodies of anti-caveolin-1, anti-ERK1/2 and anti-phospho-ERK1/2 (Santa Cruz Bio-technology, USA), followed by a 1:1000 dilution of a horseradish peroxidase (HRP)-conjugated goat anti-mouse or goat anti-rabbit IgG secondary antibody (Boster Biotechnology, Wuhan, China). Densitometry of scanned films was performed with Alphalmage 2200 Image software (UVP Company, USA).

Caveolin-1 knock down. The expression of caveolin-1 in VSMC was suppressed by specific caveolin-1 small interfering RNA (siRNA) whose sequence was 5'-CUUAAACCCUCAAGCAGAUC-3' (Sangon Biological Engineering Technology, Shanghai, China). VSMC were seeded at 5 × 10⁵ cells per well into six-well plates and allowed to adhere for 24 h. The cells were treated with 100 nM caveolin-1 siRNA using Lipofectamine 2000 (Invitrogen, Inc., Carlsbad, USA) as the transfection reagent for 24 h. Only transfection reagent only or scrambled siRNA 5'-UAGCGACUAAACACUCAA-3' (Sangon Biological Engineering Technology, Shanghai, China) were used as negative and scramble controls, respectively. The cells were treated with 120 mm Hg for 24 h for cell counting or four hours for Western blot analysis.

Disruption of ERK1/2 signal transduction. PD98059 (Sigma, USA), an inhibitor of ERK1/2 phosphorylation, was dissolved in DMSO. VSMC were pre-incubated in the absence or presence of 25 μM PD98059 for 20 min and then treated without or with 120 mm Hg for 4 h for Western blot analysis, or for 24 h for trypan blue staining.

Statistical analyses. All experiments were performed in triplicate. The results were expressed as means ± SEM, and were analyzed by t-test with SPSS 11.0. A value of p < 0.05 was considered to be statistically significant.

Results

Effect of static pressures on VSMC proliferation, ERK1/2 phosphorylation and caveolin-1 expression

MTT assay and trypan blue staining method were used to determine the effect of static pressures on cell viability and growth rate. Angiotensin II (Ang II) was a positive control and atmosphere pressure (0 mm Hg) was a negative control. As shown in Fig. 1A, both cell viability and cell number increased with the elevation of static pressure and reached their peaks at 120 mm Hg, 1.78 and 1.82 folds of that at atmosphere pressure. These results showed that static pressure stimulates the proliferation of VSMC in a biphasic pressure-dependent manner.

To explore the mechanism of static pressure-induced VSMC proliferation under static pressure, we detected the levels of caveolin-1 expression and ERK1/2 phosphorylation (Fig. 1B). We found that the expression of caveolin-1 was reduced gradually with static pressure elevation, and it reaches the lowest level at the pressure of 120 mm Hg. In contrast, ERK1/2 phosphorylation was increased gradually and reached the peak level of 120 mm Hg.

VSMC proliferation under timed static pressure was also analyzed. As shown in Fig. 1C, under the culture condition of the pressure of 120 mm Hg, the viability and total cell number of VSMC gradually increased and reached a plateau after four hours. As shown in Fig. 1D, at 4-h time point the p-ERK1/2 increased to its highest level and caveolin-1 gradually decreased to its lowest level.

Effect of static pressure on S and G2/M phases on cell cycle

VSMC were, respectively, cultured under atmosphere pressure for 24 h, or under at 120 mm Hg for 4 h or 120 mm Hg for 24 h. VSMC were analyzed by flow cytometry for cell cycles. As shown in Fig. 2, static pressure significantly drove VSMC from G0/G1 phases to S and G2/M phases. Proliferation index (PI) is the percentage of the cells at the S and G2/M phases in total cells. Pls in
shown in Fig. 3, static pressure increased ERK1/2 phosphorylation for 20 min at atmosphere pressure and then cultured in the presence of PD98059 (25 μM) to inhibit ERK1/2 phosphorylation. (A) VSMC were subjected to serum-free starvation for 24 h, and then treated with different pressures of 0, 60, 90, 120, 150, 180 mm Hg or Ang II (the final concentration of 10^{-6} M) under atmosphere pressure for 24 h. Cell viability and numbers were detected, respectively, by MTT assay and Trypan blue staining method. (B) Western blot data showed the levels of caveolin-1 and p-ERK1/2 in VSMC under different static pressures. (C) VSMC were subjected to serum-free starvation for 24 h, and then treated with 120 mm Hg for 24 h, and then treated with 120 mm Hg for 0, 2, 4, 8, 12, 24 h. Cell viability and numbers were detected, respectively, by MTT assay and Trypan blue staining method. (D) VSMC were subjected to serum-free starvation for 24 h, and then treated with 120 mm Hg for 0, 2, 4, 8, 12, 24 h. Total cellular protein was extracted for detecting caveolin-1 and p-ERK1/2 by Western blot. The results were shown as ±SEM of triplicate experiment. * < 0.01 versus Control; * * < 0.05 versus Control; * * * < 0.01 versus the group without PD98059 at 120 mm Hg for 4 h.

Groups of 120 mm Hg for 24 h and 120 mm Hg for 4 h were 30.1% and 32.4%, respectively, much higher than the control group in which the PI was 7%. Our results demonstrate that static pressure promotes significantly VSMC mitosis.

**Effect of PD98059 an inhibitor of ERK1/2 phosphorylation and VSMC proliferation**

To explore the effect of ERK1/2 phosphorylation on static pressure-driven proliferation, VSMC were pre-treated with PD98059 for 20 min at atmosphere pressure and then cultured in the pressure-adjustable incubator for 4 h at a pressure of 120 mm Hg. As shown in Fig. 3, static pressure increased ERK1/2 phosphorylation and promoted VSMC proliferation. However, the VSMC prolifera-

**Fig. 2.** Static pressure advance cell cycle. VSMC were subjected to serum-free starvation for 24 h, and then cultured, respectively, at atmosphere pressure for 24 h (control), 120 mm Hg for 4 h, and 120 mm Hg for 24 h. The cell cycles were detected by flow cytometry. The percentages of G0/G1, S, G2/M phages and the PI value (PI = percentage of S phase + percentage of G2/M phage) were calculated. The results were shown as ±SEM of triplicate experiment. * < 0.01 versus Control group; * * < 0.01 versus the group of 120 mm Hg for 4 h.

**Effect of caveolin-1 Knock down on VSMC proliferation and ERK1/2 phosphorylation**

To determine the role of caveolin-1 in static pressure-induced VSMC proliferation, endogenous caveolin-1 was knocked down by specific siRNA in VSMC. Caveolin-1 siRNA decreased endogenous caveolin-1 expression approximately threefold compared with the scrambled control and negative control (Fig. 4 A). As shown in Fig. 4B, p-ERK1/2 was significantly increased in the condition was reduced when ERK1/2 phosphorylation was inhibited by the presence of PD98059. The static pressure, VSMC proliferation and p-ERK1/2 showed a positive correlation suggesting that PD98059 blocked the ERK1/2 activation pathway.

**Fig. 3.** Blocking ERK1/2 phosphorylation reduces static pressure-induced VSMC proliferation. VSMC were pre-incubated in the absence (control) or presence of PD98059 (25 μM/l) at atmosphere pressure for 20 min. (A) VSMC were pre-treated with PD98059 (25 μM/l), continued to be treated with or without 120 mm Hg for 4 h, and then the amount of VSMC were determined by Trypan blue staining method. The results were shown as ±SEM of triplicate experiment. * < 0.01 versus the group without PD98059 at 120 mm Hg at atmosphere pressure (0 mm Hg); * * < 0.05 versus the group without PD98059 at 120 mm Hg for 24 h.
ditions of both atmosphere and 120-mm Hg pressure when the caveolin-1 expression was knocked down by siRNA. Moreover, p-ERK was upregulated more and caveolin-1 was downregulated more in the condition of 120 mm Hg than in the condition of atmosphere whether VSMC was treated without or with caveolin-1 siRNA. Both the expression of caveolin-1 and p-ERK1/2 showed pressure correlation. The silence of caveolin-1 promoted VSMC proliferation, especially at static pressure of 120 mm Hg (Fig. 4C).

Discussion

VSMC proliferation is involved in the common pathogenesis of hypertension, atherosclerosis and restenosis after PTA [1–4]. VSMC proliferation is involved in the common pathogenesis of hypertension, atherosclerosis and restenosis after PTA [1–4]. VSMC proliferation is involved in the common pathogenesis of hypertension, atherosclerosis and restenosis after PTA [1–4]. VSMC proliferation is involved in the common pathogenesis of hypertension, atherosclerosis and restenosis after PTA [1–4]. VSMC proliferation is involved in the common pathogenesis of hypertension, atherosclerosis and restenosis after PTA [1–4]. VSMC proliferation is involved in the common pathogenesis of hypertension, atherosclerosis and restenosis after PTA [1–4].

Hg). The results were shown as ±SEM of triplicate experiment.* were determined by Trypan blue staining method. 1 represents the cell number in without caveolin-1 siRNA for 4 h at atmosphere pressure (control) or at 120 mm Hg pressure correlation. The silence of caveolin-1 promoted VSMC proliferation, especially at static pressure of 120 mm Hg (Fig. 4C).

Shear stress induces cellular proliferation by activating ERK1/2 phosphorylation [26]. To investigate the mechanism of VSMC proliferation by static pressure stimulation, ERK1/2 phosphorylation was shown in both pressure- and time-dependent manners (Fig. 1). This means that the ERK1/2 signal pathway is involved in static pressure-induced VSMC proliferation. The results indicated that ERK1/2 activation occurs in the earlier phase of pressure treatment and VSMC are very sensitive to static pressure. Static pressure-induced VSMC proliferation was blocked by the exposure in 25 μM of PD98059, which is an inhibitor of ERK1/2 (Fig. 3). However, the proliferation did not show a complete shutdown, which indicated that other signal pathways may be involved in static pressure-induced VSMC proliferation. It has been well documented that shear stress stimulates the proliferation of VSMC by multiple signal pathways such as PKC, ERK1/2, p38 and so on [3]. Hishikawa et al. [27] also reported that low static pressure (80 mm Hg) could promote DNA synthesis in VSMC by the activation of PLC and PKC. These pathways may also contribute to static pressure-induced VSMC proliferation.

Caveolin-1 is a tumor suppressor gene and a negative regulator of the Ras–ERK1/2 cascade [28]. Downregulation of caveolin-1 protein expression up-regulates ERK1/2 activity in NIH3T3 cells and leads to constitutive activation of MEK and ERK1/2 [29]. These results are consistent with our current findings that downregulation of caveolin-1 expression by caveolin-1 siRNA constitutively activates signaling from MEK1/2 (Fig. 4A and B), and it correlates with VSMC proliferation (Fig. 4C). The relationships between the expression of caveolin-1, ERK1/2 and VSMC proliferation are shown in Fig. 1B (pressures) and also in Fig. 1D (times). Static pressure-driven VSMC proliferation is modulated by the caveolin-1/ERK1/2 pathway. Therefore, caveolin-1 might be an effective target gene to delay vascular sclerosis and restenosis.

In this study, we found that static pressure stimulates VSMC proliferation and that it is modulated via the caveolin-1/ERK1/2 pathway. This finding will assist us to better understand the essence of human vascular proliferative diseases and help us to discover a way to stop them.

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