Static Pressure Promotes Rat Aortic Smooth Muscle Cell Proliferation via Upregulation of Volume-Regulated Chloride Channel

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Key Words

Static pressure • Aortic smooth muscle cell • Proliferation • CIC-3 • Volume-regulated chloride channel

Abstract

Arterial smooth muscle cell proliferation is a key event in the development of hypertension associated vascular disease. Although previous studies have found that pressure itself can promote cell proliferation and DNA synthesis in vascular smooth muscle cells, the mechanisms are not clear. Recent accumulating evidence indicate that volume-regulated chloride channel plays an important role in the regulation of cell proliferation induced by numerous mitogenic factors. However, whether volume-regulated chloride channel is involved in hypertension-induced vascular smooth muscle cell proliferation remains to be determined. In this study, we found that static pressure promoted rat aortic smooth muscle cell proliferation and cell cycle progression. Static pressure treatment increased volume-regulated chloride currents and CIC-3 expression. Inhibition of chloride channel with pharmacological blockers or knockdown of CIC-3 with CIC-3 antisense transfection attenuated pressure-

evoked cell proliferation and cell cycle progression. Static pressure enhanced the production of reactive oxygen species (ROS) in aortic smooth muscle cells. Diphenyleneiodonium (DPI) or apocynin pretreatment inhibited pressure-induced ROS production as well as cell proliferation. Furthermore, DPI or apocynin attenuated the pressure-induced upregulation of CIC-3 protein and hypoosmolarity-activated chloride current. Our data suggest that volume-regulated chloride channel plays a critical role in static pressure-induced cell proliferation and cell cycle progression, suggesting the therapeutic importance of volume-regulated chloride channel for treatment of hypertension attendant vascular complications.

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Introduction

Volume-regulated chloride channel is ubiquitously distributed in mammalian cells and is thought to play an important role in many cellular physiological functions,

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including the regulation of cell volume, proliferation, differentiation and apoptosis [1-3]. Although recent studies on the molecular identity of volume-regulated chloride channel were inconsistent, CIC-3, a member of voltage-gated CIC chloride channel family, is considered as one of the key component of volume-regulated chloride channel in vascular smooth muscle cells [4-6]. Numerous growth factors, such as endothelin-1, epidermal growth factor or interleukin 1 β , have been reported to increase the expression of CIC-3 chloride channel [7-9]. Knockdown of CIC-3 with specific antisense oligonucleotides or siRNA transfection inhibited cell proliferation and cell cycle progression induced by several different kinds of growth factors [1, 9, 10]. Moreover, the study in CIC-3 knockout mice demonstrated that the proliferation of vascular smooth muscle cells from *Cln3*^{-/-} mice was slower than those from *Cln3*^{+/-} littermate [11]. These results provided evidence that CIC-3 chloride channel plays a crucial role in cell proliferation induced by some mitogenic factors.

Vascular remodeling is a prominent feature of chronic hypertension and is considered as one of the major contributors to cardiovascular diseases [12-14]. Abnormal vascular smooth muscle cell growth contributes to vascular remodeling [12, 15]. In hypertension, proliferation of vascular smooth muscle cells is caused by multi-factors, including growth factors, cytokines and pressure itself [16-20]. Interestingly, one recent study [21] reported that the volume-regulated chloride movement is increased in hypertensive rat basilar smooth muscle cells and its increment parallels the severity of hypertension, suggesting volume-regulated chloride channel may be involved in vascular smooth muscle cell proliferation and vascular remodeling during hypertension. However, whether this channel contributes to the pressure itself induced vascular smooth muscle cell proliferation remains unknown.

Therefore, in this study, we investigated the functional role of volume-regulated chloride channel in pressure-induced proliferation of rat aortic smooth muscle cells. Our findings demonstrated that static pressure treatment increased hypotonicity-activated chloride current and CIC-3 expression. Inhibition of chloride channel with pharmacological blockers or knockdown of CIC-3 attenuated pressure-evoked cell proliferation and cell cycle progression. The production of reactive oxygen species underlies the pressure-induced upregulation of volume-regulated chloride channel in aortic smooth muscle cells.

Materials and Methods

Materials

Cell culture supplies were purchased from Gibco (Grand Island, NY, USA). Anti-CIC-3 antibody was supplied by Alomone Labs (Jerusalem, Israel). α -actin monoclonal antibody was purchased from Santa Cruze. BrdU Assay Kit was purchased from Invitrogen. Diphenyleneiodonium (DPI), apocynin, 3-(4,5-dimethylthiazol-2-yl)2,5-Diphenyltetrazolium bromide (MTT), Trypsin, CsCl, CsOH, ATP-Mg, N-methyl-D-glucamine (NMDG), tetraethylammonium chloride (TEACl), N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), 5-nitro-2-[3-phenylpropylamino] benzoic acid (NPPB) and 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS) were obtained from Sigma.

Pressure-Loading Apparatus

A glass bottle was used as the pressure-loading apparatus as previously described [22]. During the experiments, the cultures in flasks with vent caps were placed in the bottle. The glass bottle was then sealed with a silicon plug which was connected to a manometer and pressurized at 80, 120 or 160 mmHg. The connector was then clamped and the glass bottle was then incubated at 37°C. The gas in the bottle was replaced and the pressure amplitude was adjusted every 6 hours to maintain the pressure and to prevent hypoxia. In our experiments, HIF-1 α expression of aortic smooth muscle cells cultured in the glass for 6 hours was not different from that cultured under pressure-free condition (1.13 $\pm$ 0.21-fold vs. pressure-free condition).

Cell culture

Rat aortic smooth muscle cells were grown from the aortic explants of male Sprague-Dawley rats (180-250 g) and were cultured in DMEM/F12 medium with 10% fetal calf serum, 50 μ g/ml streptomycin and 50 U/ml penicillin as previously described [23]. The cultures were maintained at 37°C in a humidified incubator in a 95% O₂ plus 5%CO₂ atmosphere. The cells were identified as smooth muscle cells by the morphological characteristics and by the positive response to the monoclonal antibody specific for smooth muscle α -actin.

5-bromo-2'-deoxyuridine (BrdU) incorporation

Cell proliferation was assessed by BrdU incorporation as previously reported [10]. Briefly, BrdU were added into the cells and incubated for 18 h. After fixation, the cells were treated with anti-BrdU primary antibody for 1 h at room temperature. After incubation with horseradish peroxidase-conjugated goat anti-IgG for 30 min, 3,3',5,5'-tetramethylbensidine was then added as the substrate for horseradish peroxidase. The incorporation was measured at 450 and 540nm on an Elisa microplate reader (BIO-TEK, American).

Flow cytometry for cell cycle analysis

After treatment, aortic smooth muscle cells were collected by centrifugation at 1,000 rpm for 5 min at 4°C. Pellets were rinsed once with ice-cold PBS and fixed with 70% ethanol for 24 h. Then the samples were stained with staining buffer (PBS

Fig. 1. Cl^- channel blockers inhibited static pressure-induced rat aortic smooth muscle cell proliferation. A and B, static pressure induced aortic smooth muscle cell proliferation in pressure-dependent (A) and time-dependent (B) manners ($n=8$). C to E, Cl^- channel blockers DIDS (10–100 μM) (D, $n=6$) and NPPB (10–100 μM) (E, $n=7$) inhibited static pressure-induced rat aortic smooth muscle cell proliferation in a concentration-dependent manner. However, DIDS (100 μM) and NPPB (100 μM) had no effects on BrdU incorporation under normal pressure (C, $n=4$). # $p<0.05$ compared with corresponding control group. * $p<0.05$ compared with corresponding pressure only group.

containing 50 $\mu\text{g}/\text{ml}$ of propidium iodide, 10 $\mu\text{g}/\text{ml}$ RNase A, 0.1% sodium citrate and 0.1 TritonX-100). DNA content was analyzed by flow cytometry (EPICS XL, BECKMAN COULTER, USA) to determine the population fractions in different cell cycle phases.

CIC-3 antisense oligonucleotides transfection

The antisense and sense oligonucleotides of CIC-3 were synthesized (Sangon, Shanghai, China) as reported previously [9]. The antisense sequence was 5'-TCC ATT TGT CAT TGT-3'. The sense oligonucleotide sequence was 5'-ACA ATG ACA AAT GGA-3' and was used as the control. The oligonucleotides were transfected with lipofectAMINE²⁰⁰⁰ (5 $\mu\text{l}/\text{ml}$) according to the manufacture's instructions. In brief, aortic smooth muscle cells were plated onto 96-well ViewPlates (for superoxide measurements and MTT assay) or 35-mm culture dishes (for cell cycle analysis and protein extraction). Before transfection, cells were washed with serum- and antibiotic-free DMEM and were incubated in serum- and antibiotic-free DMEM containing 5 $\mu\text{l}/\text{ml}$ lipofectAMINE²⁰⁰⁰ (Invitrogen, USA) complexed with antisense (100 $\mu\text{g}/\text{ml}$) or sense (100 $\mu\text{g}/\text{ml}$) oligonucleotides. After 6h incubation at 37°C, the cells were then incubated in DMEM containing 10% FBS for another 48 h before experiments.

Western blot

Western blot analysis of CIC-3 expression was performed as we described previously [24]. Briefly, 50 μg of protein extracts from specific samples were separated by 8% SDS-PAGE and transferred to PVDF membranes (millipore). The membranes were then incubated with rabbit anti-CIC-3 antibody for 1 hour at room temperature. The immunoblots were detected with horse-radish peroxidase-conjugated goat anti-rabbit IgG for 1 hour at room temperature. The blots were developed with an enhanced chemiluminescence detection kit (Amersham Biosciences) and then visualized by exposure to Kodak X-ray film.

Electrophysiological experiments

The whole-cell membrane Cl^- currents were recorded with an Axopatch 200A Amplifier (Axon instrument, Foster City, CA, U.S.A.) as previously described [6]. The cells were bathed at room temperature (22–25°C) and continuously superfused with isotonic or hypotonic solutions. Patch pipettes with resistances of 3–5 Ω were made from borosilicate glass using a two-stage puller (pp-830, Narishige, Tokyo, Japan). An agar bridge was used to minimize the changes of liquid junctional potentials. To determine the whole-cell current-voltage relationship, cell was held at -40mV, and test potentials were applied from -100mV to +120 mV for 400ms in +20mV increments at an interval of 5s. Currents were filtered at a frequency of 2 kHz and digitized at 5 kHz using pCLAMP7.0 software (Axon instruments). Current densities were calculated by normalizing the current amplitude to membrane capacitance.

The isotonic bath solution (300 mosmol/kg.H₂O) contained (mM): 107 N-methyl- D-glucamine chloride (NMDG-Cl), 1.5 MgCl_2 , 2.5 MnCl_2 , 0.5 CdCl_2 , 0.05 GdCl_3 , 10 glucose, 10 Hepes and 70 mM D-Mannitol, pH 7.4 adjusted with NMDG. The 230 mosmol/kg.H₂O hypotonic solution were made by omitting D-mannitol in isotonic solution. The pipette solution contained (mM): 95 CsCl, 20 TEACl, 5 ATP-Mg, 5 EGTA, 5 Hepes, and 80 D-mannitol, pH 7.2 adjusted by CsOH.

Measurement of Reactive Oxygen Species Production

ROS production was assessed by using 2,7-dichlorofluorescein diacetate (DCF-DA) [25]. Cells were grown in 96-well plates and were subjected to different treatments (see "Results"), the cells were then washed with HBSS (mM: NaCl 130, KCl 5, MgCl_2 1, CaCl_2 1.3, HEPES 20, glucose 10, pH 7.4) and loaded with 50 μM DCF-DA dissolved in HBSS for 30min at 37 °C. Subsequently, DCF fluorescence was measured with a multiwell fluorescence plate reader (Fluorostar BMG, Offenburg, Germany).

Fig. 2. Cl^- channel blockers suppressed static pressure-induced cell cycle transition in rat aortic smooth muscle cells. A and B, static pressure (80-160 mmHg) treatment for 48 h promoted the cell cycle transition from G0/G1 phase to S phase in a pressure-dependent manner (n=6). C, Cl^- channel blockers DIDS (100 μM) and NPPB (100 μM) remarkably inhibited the pressure-induced cell cycle transition (n=6). * $p<0.05$ compared with corresponding control group. # $p<0.05$ compared with normal pressure group.

Statistical analysis

All data are represented as means $\pm$ SEM. Statistical significance is tested by Student's t test or one-way analysis of variance (ANOVA). $p<0.05$ was considered significant.

Results

Cl^- channel blockers inhibited static pressure-induced aortic smooth muscle cell proliferation

Static pressure stimulated rat aortic smooth muscle cells proliferation in pressure- and time-dependent manners. The maximal increase was observed at 120 mmHg after 48 h treatment (Fig. 1A, B). So we chose 120 mmHg treatment for 48 h to do our following cell proliferation experiment. Cl^- channel blockers DIDS (10-100 μM) and NPPB (10-100 μM) significantly suppressed pressure-induced cell proliferation in a concentration-dependent manner (Fig. 1C, to 1E).

Cl^- channel blockers prevented pressure-promoted cell cycle progression

After synchronization for 24 h in DMEM/F12 medium with 0.2% fetal calf serum, aortic smooth muscle cells were mostly arrested in G0/G1 phase (77.6 $\pm$ 8.4%). Static pressure (80-160 mmHg) treatment for 48 h promoted the cell cycle transition from G0/G1 phase to S phase in a pressure-dependent manner (Fig. 2A and 2B). Cl^- channel blockers DIDS (100 μM) and NPPB (100 μM) remarkably inhibited the pressure-induced cell cycle transition (Fig. 2C).

Static pressure altered the expression of CDK2 and $p27^{\text{Kip1}}$, $p21^{\text{WAF1/CIP1}}$

Cell cycle progression is governed by the interaction of cyclins, cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CDKIs). We, therefore, determined the expression of proteins regulating the cell-cycle progression after static pressure treatment. Our results demonstrated that static pressure increased the expression of CDK2 and decreased the expression of two CDKIs, $p27^{\text{Kip1}}$ and $p21^{\text{WAF1/CIP1}}$ in rat aortic smooth muscle cells (n=3-5, Fig. 3).

Static pressure increased hypotonicity-activated Cl^- current in aortic smooth muscle cells

Whole-cell Cl^- current was recorded in control and in pressure-treated cells. After 48 h of pressurization at 120 mmHg, the basal current amplitude in the isotonic solution showed a slight but significant increase. Hypotonic solution evoked an outward rectifying current both in control and in pressure-treated cells with the reversal potentials of $-1.6\pm2.4\text{mV}$ and $-2.3\pm1.8\text{mV}$, respectively, which is close to the equilibrium potential of Cl^- . The current amplitudes at +100 mV and -100 mV in cells pressurized at 120 mmHg for 48 h were $77.9\pm6.3\text{ pA/pF}$ and $-39.4\pm4.2\text{ pA/pF}$ (n=8), which were significantly larger than those in control group ($42.3\pm3.3\text{ pA/pF}$ and $-24.0\pm2.7\text{ pA/pF}$ at +100 mV and -100 mV, respectively. $p<0.05$, n=7) (Fig. 4).

Fig. 3. Effects of static pressure on the expression of cell cycle related proteins. Left panel, representative western blot images of CDK2 (A) and cyclin-dependent kinase inhibitors (CDKIs), p27^{Kip1} (B) and p21^{WAF1/CIP1} (C). Right panel, densitometric analysis showed that static pressure increased the expression of CDK2 (A, n=5) and decreased the expression of p27^{Kip1} (B, n=3) and p21^{WAF1/CIP1} (C, n=3). * P<0.05, **P<0.01 compared with corresponding control group.

Fig. 4. Static pressure increased hypotonicity-activated Cl⁻ current in rat aortic smooth muscle cells. Whole-cell Cl⁻ current was recorded in control and in static pressure-treated (120 mmHg for 48 h) cells. A, representative traces of Cl⁻ currents from control and pressure-treated group in isotonic and hypotonic solutions. B, I-V curves of volume-regulated Cl⁻ current from control group (n=7) and pressure-treated group (n=8). C, the mean current densities measured at +100 mV and -100 mV from control group (n=7) and pressure-treated group (n=8). *p<0.05 compared with corresponding control group.

Static pressure increased endogenous CIC-3 expression

Recent study in A10 vascular smooth muscle cells has implicated the important role of CIC-3 in the regulation of hypotonicity-activated Cl⁻ efflux and Cl⁻ current. We then examined whether static pressure could affect endogenous CIC-3 expression in aortic smooth muscle cells by western blotting using a polyclonal antibody directed against CIC-3. As shown in Figure 5A, static pressure stimulated the expression of CIC-3 protein in a pressure-dependent manner (n=5).

To further determine the relationship between CIC-3 and volume-regulated chloride channel in rat aortic smooth muscle cells, we tested the effect of CIC-3 knock-down with specific CIC-3 antisense oligonucleotides transfection on pressure-induced increase of volume-regulated chloride current. Figure 5B showed that 100 µg/ml CIC-3 antisense oligonucleotides transfection remarkably decreased pressure-induced CIC-3 upregulation in rat aortic smooth muscle cells. However, sense oligonucleotides or lipofectAMINE²⁰⁰⁰ (lipo) transfection exerted no effect on CIC-3 expression.

Fig. 5. Knockdown of CIC-3 inhibited the pressure-induced increase of volume-regulated chloride current in rat aortic smooth muscle cells. A, static pressure increased endogenous CIC-3 expression in a pressure-dependent manner (n=5). B, CIC-3 antisense oligonucleotide (100 μ g/ml) transfection (Anti) inhibited the static pressure-induced (120 mmHg) CIC-3 expression. Sense oligonucleotide or lipofectAMINE²⁰⁰⁰ (Lipo) treatment had no effects on CIC-3 expression (n=6). C, I-V curves of hypotonicity-activated Cl⁻ current from control group (n=8) and CIC-3 antisense transfection group (anti, n=9) after the cells were pressurized at 120 mmHg for 48 h. D, the mean current densities measured at +100 mV and -100 mV from control group (n=7), pressure treatment group (n=8) and pressure treatment cells transfected with CIC-3 antisense (n=9) or sense oligonucleotides (n=6) or lipofectAMINE²⁰⁰⁰ (Lipo) (n=6). #p<0.05 compared with corresponding control group, *p<0.05 compared with corresponding pressure only group.

Fig. 6. Downregulation of CIC-3 attenuated pressure-induced aortic smooth muscle cell proliferation and cell cycle progression. A, downregulation of CIC-3 expression with antisense oligonucleotides transfection significantly attenuated static pressure-induced cell proliferation (n=7). B, CIC-3 knockdown markedly inhibited pressure-induced cell cycle transition from G0/G1 phase to S phase (n=6). #p<0.05 compared with corresponding control group, *p<0.05 compared with corresponding pressure only group.

Moreover, CIC-3 antisense oligonucleotides transfection reversed the increased hypoosmolarity-activated chloride current in pressurized cells. CIC-3 knockdown decreased the current amplitudes from 77.9 ± 6.3 pA/pF and -39.4 ± 4.2 pA/pF (n=8) to 47.7 ± 4.1 pA/pF and -27.6 ± 1.8 pA/pF (n=9, p<0.05) at +100 mV and -100 mV, respectively, in aortic smooth muscle cells pressurized at 120 mmHg for 48 h (Fig. 5C and 5D), indicating that CIC-3 upregulation underlies the pressure-induced increases of volume-regulated chloride currents in rat aortic smooth muscle cells.

Downregulation of CIC-3 attenuated pressure-induced cell proliferation and cell cycle progression

The functional role of volume-regulated chloride channel in the regulation of static pressure-induced cell

proliferation was further evaluated in aortic smooth muscle cells in which CIC-3 expression was reduced by transfection of CIC-3 antisense oligonucleotides. Our results showed that downregulation of CIC-3 significantly inhibited pressure-induced cell proliferation and cell cycle transition from G0/G1 phase to S phase (Fig. 6A and Fig. 6B).

Static pressure induced aortic smooth muscle cell proliferation via reactive oxygen species (ROS) production

Figure 7A demonstrated that static pressure increased the ROS production in aortic smooth muscle cells. Pretreatment with DPI (100 μ M) or apocynin (10 μ M) reduced the pressure induced ROS production. Moreover, DPI or apocynin preincubation remarkably suppressed the pressure-induced cell proliferation (Fig. 7B). These

Fig. 7. NADPH derived reactive oxygen species (ROS) production mediated the pressure-induced aortic smooth muscle cell proliferation. A, static pressure increased the ROS production in aortic smooth muscle cells. Pretreatment with DPI (100 μ M) or apocynin (10 μ M) reduced the pressure-induced ROS production (n=5). B, DPI (100 μ M) or apocynin (10 μ M) preincubation remarkably suppressed the pressure-induced cell proliferation (n=6). #p<0.05 compared with corresponding control group, *p<0.05 compared with corresponding pressure only group.

Fig. 8. Static pressure-induced ROS production mediated the upregulation of volume-regulated chloride channel in rat aortic smooth muscle cells. A, DPI (100 μ M) or apocynin (10 μ M) pretreatment reversed pressure-evoked upregulation of CIC-3 protein (n=5). B, DPI (100 μ M) or apocynin (apo, 10 μ M) preincubation attenuated pressure-induced increase of volume-regulated chloride current. C, The current amplitudes measured at +100 mV and -100 mV in control group, pressure-treated groups with or without DPI or apocynin. #p<0.05 compared with corresponding control group, *p<0.05 compared with corresponding pressure only group.

results indicated the NADPH oxidase-derived ROS production mediated the pressure-induced aortic smooth muscle cell proliferation.

Static pressure-induced ROS production mediated the upregulation of volume-regulated chloride channel

To determine whether the pressure-induced ROS production mediated the upregulation of volume-regulated chloride channel, we investigated the effects of DPI or apocynin on CIC-3 expression and volume-regulated chloride current in aortic smooth muscle cells. Our results showed that DPI (100 μ M) or apocynin (10 μ M) pretreatment reversed the pressure-evoked upregulation of CIC-3 protein and hypoosmolarity-activated chloride current (Fig. 8A and 8B). At +100 mV and -100 mV, DPI or apocynin treatment decreased the current amplitudes in pressure-treated group from

78.7 $\pm$ 8.4 pA/pF and -41.3 $\pm$ 5.7 pA/pF (n=6) to 50.8 $\pm$ 4.7 pA/pF and -28.8 $\pm$ 3.1 pA/pF (n=7, p<0.05) or to 46.4 $\pm$ 5.4 pA/pF and -25.9 $\pm$ 3.3 pA/pF (n=6, p<0.05), respectively (Fig. 8C). These data suggested that NADPH oxidase-derived ROS production mediate the pressure-induced upregulation of volume-regulated chloride channel in aortic smooth muscle cells.

Discussion

Our results in this study demonstrated static pressure-induced rat aortic smooth muscle cell proliferation and cell cycle progression were significantly suppressed by Cl⁻ channel blockers. Moreover, static pressure increased the expression of CIC-3 Cl⁻ channel as well as the amplitude of hypotonicity-activated Cl⁻ current. Downregulation of CIC-3 Cl⁻ channel with CIC-3 antisense transfection

remarkably reduced static pressure-evoked cell proliferation and cell cycle transition. These data indicated that ClC-3 Cl⁻ channel is involved in static pressure-induced aortic smooth muscle cell proliferation.

Hypertension is one of the most powerful risk factors in the prediction of cardiovascular disease [26, 27]. It has been reported that exposure vascular smooth muscle cells to increased pressure *per se* could increase the thickness of vascular wall *in vivo* and cell proliferation *in vitro* [18, 22, 28, 29], suggesting the increased intravascular forces during hypertension are associated with remodeling of vascular smooth muscle cells. In agreement with these studies, our present results showed that static pressure *per se* could induce proliferation of rat aortic smooth muscle cells. To understand the mechanisms underlying static pressure-induced cell proliferation, we investigated the effects of static pressure on cell cycle, and found that static pressure promotes cell cycle transition from G0/G1 to S phase by increasing the expression of CDK2 and decreasing the expression of p27^{Kip1} and p21^{WAF1/CIP1}. In this study, the increased pressure seems to be the predominant factor to induce vascular smooth muscle cell proliferation because previous study have reported that the pH value of the culture medium was maintained constant during the experiment, and mildly increased pO₂ had no significant effect on cell proliferation [18].

During the early phase of cell proliferation, cells undergo a significant increase in cell size caused by water influx that accompanies the uptake of nutrients necessary for cell metabolism. An increase in cell volume will initiate the regulatory volume decrease (RVD) process through activation of ion (K and Cl⁻) channels and transporters, which activates K and Cl⁻ efflux to normalize the cell size [30-32]. Volume-regulated chloride channel has been considered as one of the major regulators of RVD process. Recent studies have reported that the activity of volume-regulated chloride channel varies with the cell cycle phase, being low in G0 and S phases and high in G1/S phase [33, 34]. Cl⁻ channel blockers such as DIDS and NPPB could arrest cell growth in G0/G1 phase and cell proliferation induced by mitogenic factors or cytokines in rat aortic and porcine coronary vascular smooth muscle cells, human cervical cancer cells, hepatocytes and fibroblasts [1, 3]. These results demonstrated that volume-regulated chloride channel plays a critical role in the regulation of cell proliferation. It is well established that abnormal vascular smooth muscle cell proliferation contributes to hypertension-induced vascular remodeling. One recent study [21] in the hypertensive rat basilar smooth muscle

cells showed that the activity of volume-regulated chloride channel is increased compared to that in controls and its increment paralleled the development of hypertension, suggesting that volume-regulated chloride channel may be associated with the abnormal vascular smooth muscle cell proliferation in hypertension. However, the functional role of volume-regulated chloride channel in pressure-induced vascular smooth muscle cell proliferation remains elusive. In this study, we found static pressure itself could increase the basal and hypotonicity-activated chloride currents. Cl⁻ channel blockers DIDS and NPPB inhibited pressure-induced cell proliferation and cell cycle progression in aortic smooth muscle cells. Our results indicated that volume-regulated chloride channel plays an essential role in regulation of pressure-induced aortic smooth muscle cell proliferation.

Although the molecular identity of volume-regulated chloride channel is still under debate, recent studies suggested that ClC-3 chloride channel is the most potential candidate of volume-regulated chloride, especially those from vascular smooth muscle cells [6, 35, 36]. The link between ClC-3 chloride channel and cell proliferation was firstly demonstrated in rat aortic smooth muscle cells [9]. In this study, Wang et al. found that endothelin-1 increased the expression of ClC-3 protein. Downregulation of ClC-3 with ClC-3 specific antisense oligonucleotide transfection inhibited the proliferative effect of endothelin-1 on aortic smooth muscle cells. The similar results were subsequently reported in rat basilar smooth muscle cells [10], canine pulmonary smooth muscle cells [7] and human prostate cancer epithelial cells [37]. Moreover, the study in ClC-3 knockout mice demonstrated that the proliferation of vascular smooth muscle cells from *Clcn3*^{-/-} mice was slower than those from *Clcn3*^{+/+} littermate [11]. These results indicated that ClC-3 chloride channel plays a crucial role in regulation of cell proliferation induced by mitogenic factors. More interestingly, one recent work in glioma cell and immature astrocytes demonstrated that ClC-3 chloride channel is involved in the cell division process and play an important role in regulation of cytoplasmic and DNA condensation phase of mitosis [38]. In the present study, our results showed that static pressure itself could increase the ClC-3 expression in rat aortic smooth muscle cells. ClC-3 antisense oligonucleotides transfection reversed the increased hypoosmolarity-activated chloride current in pressurized cells, indicating that ClC-3 upregulation underlies the pressure-induced increases of volume-regulated chloride currents in rat aortic smooth muscle cells. Moreover, knockdown of ClC-3 expression with ClC-3

antisense oligonucleotide inhibited static pressure-induced cell proliferation and arrested the cells at G0/G1 phase and attenuated the pressure-induced cell cycle transition. These data are consistent with one recent work in rat basilar smooth muscle cells, in which, Tang et al. reported that CIC-3 knockdown with siRNA transfection inhibited endothelin-1-induced cell cycle progression and arrested the cells at G0/G1 phase [10].

Accumulating evidence suggests that increased levels of reactive oxygen species (ROS) in the blood vessel wall contribute to vascular dysfunction in a variety of pathological processes, including atherosclerosis, diabetes, and hypertension [39-41]. The major source of ROS in vascular smooth muscle cells is NAD(P)H oxidase complex [42]. Our results in this study showed that static pressure increased the ROS production in aortic smooth muscle cells. Pretreatment with DPI, an inhibitor of flavin-containing enzymes such as NADPH oxidase [43], or apocynin, the NADPH oxidase inhibitor [25, 44], reduced the pressure induced ROS production. Moreover, the pressure-induced cell proliferation was remarkably suppressed by DPI or apocynin preincubation. Furthermore, our results demonstrated that DPI or apocynin treatment reversed the pressure-induced upregulation of CIC-3 protein and volume-regulated chloride current. These results indicated that NADPH oxidase-derived ROS plays a key role in pressure-induced vascular smooth muscle

cell growth through upregulation of volume-regulated chloride channel in aortic smooth muscle cells. Of course, our results could not exclude the possibility that ROS increased the activity of volume-regulated chloride channel by promoting the translocation of CIC-3 from the cytoplasm to cell surface as observed in human leukaemia cells [45].

In summary, we demonstrated that static pressure increased CIC-3 expression and the activity of volume-regulated chloride channel in rat aortic smooth muscle cells. Chloride channel blockers or CIC-3 knockdown reduced static pressure-induced aortic smooth muscle cell proliferation and cell cycle progression. Moreover, inhibition of ROS production attenuated static pressure evoked cell proliferation as well as the upregulation of volume-regulated chloride channel. These results indicated that ROS-volume-regulated chloride channel signaling pathway plays a critical role in pressure-induced vascular smooth muscle cell proliferation.

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References

- Guan YY, Wang GL, Zhou JG: The CIC-3 Cl⁻ channel in cell volume regulation, proliferation and apoptosis in vascular smooth muscle cells. *Trends Pharmacol Sci* 2006;27:290-296.
- Hume JR, Duan D, Collier ML, Yamazaki J, Horowitz B: Anion transport in heart. *Physiol Rev* 2000;80:31-81.
- Nilius B, Droogmans G: Amazing chloride channels: an overview. *Acta Physiol Scand* 2003;177:119-147.
- Duan D, Winter C, Cowley S, Hume JR, Horowitz B: Molecular identification of a volume-regulated chloride channel. *Nature* 1997;390:417-421.
- Hermoso M, Satterwhite CM, Andrade YN, Hidalgo J, Wilson SM, Horowitz B, Hume JR: CIC-3 is a fundamental molecular component of volume-sensitive outwardly rectifying Cl⁻ channels and volume regulation in HeLa cells and *Xenopus laevis* oocytes. *J Biol Chem* 2002;277:40066-40074.
- Zhou JG, Ren JL, Qiu QY, He H, Guan YY: Regulation of intracellular Cl⁻ concentration through volume-regulated CIC-3 chloride channels in A10 vascular smooth muscle cells. *J Biol Chem* 2005;280:7301-7308.
- Dai YP, Bongalon S, Hatton WJ, Hume JR, Yamboliev IA: CIC-3 chloride channel is upregulated by hypertrophy and inflammation in rat and canine pulmonary artery. *Br J Pharmacol* 2005;145:5-14.
- Lemonnier L, Shuba Y, Crepin A, Roudbaraki M, Slomianny C, Mauroy B, Nilius B, Prevarskaia N, Skryma R: Bcl-2-dependent modulation of swelling-activated Cl⁻ current and CIC-3 expression in human prostate cancer epithelial cells. *Cancer Res* 2004;64:4841-4848.
- Wang GL, Wang XR, Lin MJ, He H, Lan XJ, Guan YY: Deficiency in CIC-3 chloride channels prevents rat aortic smooth muscle cell proliferation. *Circ Res* 2002;91:E28-32.
- Tang YY, Liu YJ, Zhou JG, Wang GL, Qiu QY, Guan YY: Silence of CIC-3 Cl⁻ channel inhibits cell proliferation and cell cycle via G1/S phase arrest in rat basilar arterial smooth muscle cells. *Cell Prolif* 2008;41:775-785.
- Miller FJ Jr, Filali M, Huss GJ, Stanic B, Chamseddine A, Barna TJ, Lamb FS: Cytokine activation of nuclear factor kappa B in vascular smooth muscle cells requires signaling endosomes containing Nox1 and CIC-3. *Circ Res* 2007;101:663-671.
- Baumbach GL, Heistad DD: Adaptive changes in cerebral blood vessels during chronic hypertension. *J Hypertens* 1991;9:987-991.
- Mulvany MJ, Baumbach GL, Aalkjaer C, Heagerty AM, Korsgaard N, Schiffrin EL, Heistad DD: Vascular remodeling. *Hypertension* 1996;28:505-506.

- 14 Zlokovic BV: Remodeling after stroke. *Nat Med* 2006;12:390-391.
- 15 Hadrava V, Kruppa U, Russo RC, Lacourciere Y, Tremblay J, Hamet P: Vascular smooth muscle cell proliferation and its therapeutic modulation in hypertension. *Am Heart J* 1991;122:1198-1203.
- 16 Ueno H, Kanellakis P, Agrotis A, Bobik A: Blood flow regulates the development of vascular hypertrophy, smooth muscle cell proliferation, and endothelial cell nitric oxide synthase in hypertension. *Hypertension* 2000;36:89-96.
- 17 Wang Z, Rao PJ, Shillcutt SD, Newman WH: Angiotensin II induces proliferation of human cerebral artery smooth muscle cells through a basic fibroblast growth factor (bFGF) dependent mechanism. *Neurosci Lett* 2005;373:38-41.
- 18 Watase M, Awolesi MA, Ricotta J, Sumpio BE: Effect of pressure on cultured smooth muscle cells. *Life Sci* 1997;61:987-996.
- 19 Weissberg PL: Mechanisms of vascular smooth muscle cell proliferation. *Ann Acad Med Singapore* 1991;20:38-42.
- 20 Hishikawa K, Nakaki T, Marumo T, Hayashi M, Suzuki H, Kato R, Saruta T: Pressure promotes DNA synthesis in rat cultured vascular smooth muscle cells. *J Clin Invest* 1994;93:1975-1980.
- 21 Shi XL, Wang GL, Zhang Z, Liu YJ, Chen JH, Zhou JG, Qiu QY, Guan YY: Alteration of Volume-Regulated Chloride Movement in Rat Cerebrovascular Smooth Muscle Cells During Hypertension. *Hypertension* 2007;49(6):1371-1377.
- 22 Tada T, Nawata J, Wang H, Onoue N, Zhulanqige D, Ito K, Sugimura K, Fukumoto Y, Shimokawa H: Enhanced pulsatile pressure accelerates vascular smooth muscle migration: implications for atherogenesis of hypertension. *Cardiovasc Res* 2008;80:346-353.
- 23 Horio T, Kohno M, Kano H, Ikeda M, Yasunari K, Yokokawa K, Minami M, Takeda T: Adrenomedullin as a novel antimigration factor of vascular smooth muscle cells. *Circ Res* 1995;77:660-664.
- 24 Pang RP, Zhou JG, Zeng ZR, Li XY, Chen W, Chen MH, Hu PJ: Celecoxib induces apoptosis in COX-2 deficient human gastric cancer cells through Akt/GSK3beta/NAG-1 pathway. *Cancer Lett* 2007;251:268-277.
- 25 Abid MR, Kachra Z, Spokes KC, Aird WC: NADPH oxidase activity is required for endothelial cell proliferation and migration. *FEBS Lett* 2000;486:252-256.
- 26 Di Napoli M, Papa F: Inflammation, blood pressure, and stroke: an opportunity to target primary prevention? *Curr Hypertens Rep* 2005;7:44-51.
- 27 Panagiotakos DB, Kromhout D, Menotti A, Chrysoshoou C, Dontas A, Pitsavos C, Adachi H, Blackburn H, Nedeljkovic S, Nissinen A: The relation between pulse pressure and cardiovascular mortality in 12,763 middle-aged men from various parts of the world: a 25-year follow-up of the seven countries study. *Arch Intern Med* 2005;165:2142-2147.
- 28 Miyashiro JK, Poppa V, Berk BC: Flow-induced vascular remodeling in the rat carotid artery diminishes with age. *Circ Res* 1997;81:311-319.
- 29 Cappadona C, Redmond EM, Theodorakis NG, McKillop IH, Hendrickson R, Chhabra A, Sitzmann JV, Cahill PA: Phenotype dictates the growth response of vascular smooth muscle cells to pulse pressure in vitro. *Exp Cell Res* 1999;250:174-186.
- 30 Nilius B: Chloride channels go cell cycling. *J Physiol* 2001;532:581.
- 31 Shen MR, Droogmans G, Eggermont J, Voets T, Ellory JC, Nilius B: Differential expression of volume-regulated anion channels during cell cycle progression of human cervical cancer cells. *J Physiol* 2000;529 Pt 2:385-394.
- 32 Lang F, Shumilina E, Ritter M, Gulbins E, Vereninov A, Huber SM: Ion channels and cell volume in regulation of cell proliferation and apoptotic cell death. *Contrib Nephrol* 2006;152:142-160.
- 33 Klausen TK, Bergdahl A, Hougaard C, Christophersen P, Pedersen SF, Hoffmann EK: Cell cycle-dependent activity of the volume- and Ca²⁺-activated anion currents in Ehrlich letre ascites cells. *J Cell Physiol* 2007;210:831-842.
- 34 Bubien JK, Kirk KL, Rado TA, Frizzell RA: Cell cycle dependence of chloride permeability in normal and cystic fibrosis lymphocytes. *Science* 1990;248:1416-1419.
- 35 Yamazaki J, Duan D, Janiak R, Kuenzli K, Horowitz B, Hume JR: Functional and molecular expression of volume-regulated chloride channels in canine vascular smooth muscle cells. *J Physiol* 1998;507(Pt 3):729-736.
- 36 Duan D, Zhong J, Hermoso M, Satterwhite CM, Rossow CF, Hatton WJ, Yamboliev I, Horowitz B, Hume JR: Functional inhibition of native volume-sensitive outwardly rectifying anion channels in muscle cells and *Xenopus* oocytes by anti-ClC-3 antibody. *J Physiol* 2001;531:437-444.
- 37 Lemonnier L, Lazarenko R, Shuba Y, Thebault S, Roudbaraki M, Lepage G, Prevarskaya N, Skryma R: Alterations in the regulatory volume decrease (RVD) and swelling-activated Cl⁻ current associated with neuroendocrine differentiation of prostate cancer epithelial cells. *Endocr Relat Cancer* 2005;12:335-349.
- 38 Habela CW, Olsen ML, Sontheimer H: ClC3 is a critical regulator of the cell cycle in normal and malignant glial cells. *J Neurosci* 2008;28:9205-9217.
- 39 Cai H, Harrison DG: Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circ Res* 2000;87:840-844.
- 40 Nishiyama A, Fukui T, Fujisawa Y, Rahman M, Tian RX, Kimura S, Abe Y: Systemic and Regional Hemodynamic Responses to Tempol in Angiotensin II-Infused Hypertensive Rats. *Hypertension* 2001;37:77-83.
- 41 Somers MJ, Mavromatis K, Galis ZS, Harrison DG: Vascular superoxide production and vasomotor function in hypertension induced by deoxycorticosterone acetate-salt. *Circulation* 2000;101:1722-1728.
- 42 Paravicini TM, Touyz RM: Redox signaling in hypertension. *Cardiovasc Res* 2006;71:247-258.
- 43 Hancock JT, White JJ, Jones OT, Silver IA: The use of diphenylene iodonium and its analogues to investigate the role of the NADPH oxidase in the tumoricidal activity of macrophages in vitro. *Free Radic Biol Med* 1991;11:25-29.
- 44 Kashiwagi A, Shinozaki K, Nishio Y, Maegawa H, Maeno Y, Kanazawa A, Kojima H, Haneda M, Hidaka H, Yasuda H, Kikkawa R: Endothelium-specific activation of NAD(P)H oxidase in aortas of exogenously hyperinsulinemic rats. *Am J Physiol* 1999;277:E976-983.
- 45 Kasinathan RS, Foller M, Lang C, Koka S, Lang F, Huber SM: Oxidation induces ClC-3-dependent anion channels in human leukaemia cells. *FEBS Lett* 2007;581:5407-5412.