High pulsatility flow stimulates smooth muscle cell hypertrophy and contractile protein expression

Devon Scott,1,* Yan Tan,1,* Robin Shandas,2 Kurt R. Stenmark,3 and Wei Tan1,2,3

1Department of Mechanical Engineering, University of Colorado at Boulder, Boulder, Colorado; 2Department of Bioengineering, University of Colorado at Denver, The Children’s Hospital at Denver, Aurora, Colorado; and 3Department of Pediatrics, University of Colorado at Denver, The Children’s Hospital at Denver, Aurora, Colorado

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High pulsatility flow stimulates smooth muscle cell hypertrophy and contractile protein expression. Am J Physiol Lung Cell Mol Physiol 304:L70–L81, 2013. First published October 19, 2012; doi:10.1152/ajplung.00342.2012.—Proximal arterial stiffening is an important predictor of events in systemic and pulmonary hypertension, partly through its contribution to downstream vascular abnormalities. However, much remains undetermined regarding the mechanisms involved in the vascular changes induced by arterial stiffening. We therefore addressed the hypothesis that high pulsatility flow, caused by proximal arterial stiffening, induces downstream pulmonary artery endothelial cell (EC) dysfunction that in turn leads to phenotypic change of smooth muscle cells (SMCs). To test the hypothesis, we employed a model pulmonary circulation in which upstream compliance regulates the pulsatility of flow waves imposed onto a downstream vascular mimetic coculture composed of pulmonary ECs and SMCs. The effects of high pulsatility flow on SMCs were determined both in the presence and absence of ECs. In the presence of ECs, high pulsatility flow increased SMC size and expression of the contractile proteins, smooth muscle α-actin (SMA) and smooth muscle myosin heavy chain (SM-MHC), without affecting proliferation. In the absence of ECs, high pulsatility flow decreased SMC expression of SMA and SM-MHC, without affecting SMC size or proliferation. To identify the molecular signals involved in the EC-mediated SMC responses, mRNA and/or protein expression of vasoconstrictors [angiotensin-converting enzyme (ACE) and endothelin (ET)-1], vasodilator (eNOS), and growth factor (TGF-β1) in EC were examined. Results showed high pulsatility flow decreased eNOS and increased ACE, ET-1, and TGF-β1 expression. ACE inhibition with ramiprilat, ET-1 receptor inhibition with bosentan, and treatment with the vasodilator bradykinin prevented flow-induced, EC-dependent SMC changes. In conclusion, high pulsatility flow stimulated SMC hypertrophy and contractile protein expression by altering EC production of vasoactive mediators and cytokines, supporting the idea of a coupling between proximal vascular stiffening, flow pulsatility, and downstream vascular function.

vascular stiffening; pulse flow; endothelial mechanotransduction; smooth muscle hypertrophy

IT IS INCREASINGLY RECOGNIZED that stiffening of elastic arteries occurs in vascular diseases such as systemic and pulmonary hypertension (6, 12, 17, 32, 45). This may be due to the fact that, besides being a conduit between the heart and arterioles, the elastic arteries act as a cushion, transforming pulsatile flow at the large elastic arteries into steady flow through the arterioles (20, 29, 44). Normally, the tasks are so efficiently performed that mean pressure is well maintained throughout the whole arterial tree where pulsatility around the mean is minimized by the elastic arteries. Artery stiffening due to aging, diabetes, or other causes reduces the cushion or buffering function, increases pulse pressure, and extends high pulsatility flow into downstream vessels, including the microcirculation (27). Aortic stiffening has also been correlated with small artery abnormalities in organs, including the kidney, brain, and eye (15, 19, 30, 31, 60). Although recent studies have gradually brought to light some basic knowledge of the concepts as to how the modulation function of elastic arteries is disordered in vascular diseases, few have looked into the cellular and molecular effects exerted on the distal vasculature by proximal vascular stiffening.

In the case of pulmonary hypertension, proximal pulmonary arterial (PA) stiffening and distal arterial medial thickening and muscularization are two prominent pathological features (48). No previous studies have attempted to make pathogenic connections between these observations. Medial thickening in pulmonary hypertension is characterized by increased smooth muscle mass, which results from alterations in smooth muscle cell (SMC) phenotype and function, and contributes significantly to increased PA resistance (48, 55). Mature vascular SMCs express a number of smooth muscle-specific genes and proteins characteristic of their contractile and “differentiated” phenotype, including smooth muscle α-actin (SMA) and smooth muscle myosin heavy chain (SM-MHC), which contribute to the contractile function of SMC (36, 42). In the pulmonary microcirculation, smooth muscle hyperplasia or appearance of new smooth muscle in previously nonmuscular or partially muscular vessels is consistently observed in all forms of pulmonary hypertension (22, 55). The mechanisms regulating SMC hyperplasia in the small arteries of the lung phenotype of downstream SMC, are unknown.

The vascular endothelium plays critical roles in smooth muscle homeostasis and function and is capable of sensing shear stress and transducing hemodynamic forces into the production of biochemical molecules that influence vascular wall remodeling. Importantly, endothelial cells (ECs) have been shown to be capable of discriminating distinct types of flow patterns (7). Hemodynamic stresses in the vascular system are under strict regulation, i.e., there exists a narrow hemodynamic range of stresses in that small perturbation of mechanical homeostasis may lead to activation of signaling events in ECs, which alters production of cytokines to impact other cells such as SMCs in the blood vessel wall (7, 18). We sought to test the hypothesis that high pulsatility flow caused by proximal vas-
cular stiffening induces change in SMC phenotype consistent with medial thickening and distal muscularization. To explore the effects of vascular stiffening on SMCs in the distal vasculature, a mimetic flow system with vascular coculture was developed to integrate how upstream compliance regulates SMC phenotype. Proximal arterial “stiffness” was modulated and regulated with a compliance chamber that controlled pulse wave intensity. The pulse waves generated were imposed on a vascular mimetic created by embedding SMC in a three-dimensional (3-D) collagen matrix adjacent to a porous permeable membrane with a monolayer of ECs. The membrane separated cells while keeping the two cell types in close proximity for cell-cell communication. With this simplified model, we aimed to explore whether and how ECs exposed to high pulsatility flow (or decreased upstream compliance) might affect underlying SMC size, contractile protein expression, and proliferation. Our results are among the first to show mechanisms that explain the connection between the two pathological features of pulmonary hypertension, i.e., reduced upstream compliance and downstream muscularization.

METHODS

Cell culture. Bovine PA ECs and SMCs were obtained from distal bovine vascular arteries as previously described (10). These cells were maintained in Dulbecco’s modified Eagle’s medium (Cellgro DMEM; Mediatech, Manassas VA), with 10% bovine calf serum (BCS) (Gemini Bio-products, West Sacramento, CA) and 1% penicillin/streptomycin. Cell passages of 3–8 were used for all experiments. All experimental conditions used 0.1% BCS, including the static culture and flow cultures.

Vascular mimetic coculture under flow conditions. To ensure collagen matrix attachment to a glass slide for flow studies, the glass slide was treated to enhance collagen binding. Glass slides were chemically functionalized with hydroxyl groups using NaOH. Amine groups were then attached to the slide surface via reaction with 95% of 3-aminopropyltriethoxysilane (Sigma, St. Louis, MO). After silanization, an aldehyde cross-linker was attached to the amine group via reaction with 1% (vol/vol) glutaraldehyde (Sigma) in phosphate buffer. This yielded an aldehyde that can form an amine linkage with the primary amines on the macromolecular chains of collagen. The collagen matrix was polymerized on the glass slides and immobilized to the surface through a reaction between the amine group on the matrix chains near the glass slide and the aldehyde group on the surface.

SMCs (passage 3–6) were grown until confluent and added to the collagen prepolymer solution. Briefly, the prepolymer solution for the collagen matrix was made by neutralizing type I collagen solution (BD Science, San Jose, CA) with PBS and increasing its ionic strength with 7% NaHCO3 and 0.1 M of NaOH. A final collagen density of 2 mg/ml was obtained. The prepolymer solution was maintained on ice at a pH of 7.4, and SMCs were added to the solution with a density of 2 × 104 cells/ml. The cell/gel solution was mixed thoroughly and immediately transferred onto a functionalized glass slide and spread to fully cover the surface. The glass slide was then placed in the incubator for 30 min to ensure proper gelation. Finally, 0.1% BCS (serum-deprived condition) was applied to SMCs in the collagen for 72 h before the experiment to induce the SMCs into a quiescent state.

ECs (passage 3–6) were grown until confluent. ECs were seeded on a fibronectin-coated, isopore polycarbonate membrane (Millipore, Billerica, MA) with pore size of 0.8 μm. The porous membrane allowed for the separation of SMC and EC but still permits molecular signals to transfer through the pores. The porous membrane was placed in contact with the SMC-seeded collagen gel, and a second silicon gasket was placed on top of the membrane to create a flow channel where ECs were subjected to fluid shear stress. The flow was applied through a parallel plate flow chamber that was held to the gasket by a vacuum (Fig. 1A).

The flow system included a pulsatile blood pump (Harvard Apparatus, Holliston, MA) that created pulsatile flow with a systolic upstroke and a diastolic downstroke. The blood pump was connected to a compliance-adjustment chamber that acted as a hydraulic buffer to simulate changes in upstream arterial stiffness. The compliance-adjustment chamber mimicked large proximal arteries through compliance adjustment (Fig. 1B). Air in the compliance-adjustment chamber dampened the flow; the more air in the chamber, the lower level of flow pulsatility. The total volume of the air and liquid was kept constant during experimentation; thus, the level of liquid in the chamber was an indicator to the level of flow pulsatility. The cylinder of this chamber was graduated to reflect the liquid-to-air ratio, which was correlated with the pulsatility of the resultant flow waveform; a higher ratio produced a highly pulsatile waveform, and a low ratio produced nearly steady flow. The liquid/air was regulated by a pressure valve. The pressure valve was open to begin the experiment, allowing the entrance of fluid until the level of liquid reached a predetermined amount based on the desired outlet flow pulsatility. The valve was then closed to maintain a constant liquid/air ratio. Flow was measured at the inlet and outlet of the compliance chamber with a digital flowmeter (Alicat Scientific, Tucson, AZ) to determine the effect of the compliance chamber on the pulsatile waveform. The precision flowmeter was connected to an oscilloscope (Agilent Technologies, Santa Clara, CA) and a computer. Real-time flow rate and pressure were determined, and then the inlet velocity profiles were calculated. Figure 1C shows these measures for the high pulsatility flow condition. The flowmeter was only used during experimental setup. The inlet flow chamber was attached to a medium reservoir, so continuously recycled media was delivered throughout the system; the outlet of the compliance chamber was attached to the flow chamber. The outlet of the flow chamber was attached to the blood pump to complete the flow circuit.

Two flow conditions were tested: static, steady [pulsatility index (PI) = 0.2] and high pulsatility (PI = 1.7) flow. Herein, we defined the flow PI as it is commonly used in the evaluation of vascular stiffening effects and the evaluation of vascular diseases (31, 37):

\[
\text{PI} = \frac{V_{\text{max}} - V_{\text{min}}}{V_{\text{mean}}}
\]

where \(V_{\text{max}}\) is the peak systolic velocity, \(V_{\text{min}}\) is the minimum forward diastolic velocity, and \(V_{\text{mean}}\) is the average velocity. For each condition, the following two cellular setups were examined: 1) SMCs embedded in the collagen matrix without adjacent ECs and 2) coculture of EC and SMC. For all of the flow conditions, the same mean flow rate with a mean shear stress of 12 dyn/cm² was applied for 24 h. The mean flow shear used here is close to the mean resting flow shear stress measured in distal pulmonary artery in vivo (13 dyn/cm²) (51). With a constant mean velocity and frequency, a flow condition with a higher PI denotes a higher energy level (23). Regarding the design of PI condition in this study, previous studies showed that the mean flow PI in the large elastic PAs (i.e., the first two generations, main PA, left PA, and right PA) ranged from 4.4 to 5.1 in vivo, whereas the PI in the pulmonary capillaries decreased to ~1 in vivo (38, 43). In addition, our previous study with flows of several PI conditions (1, 1.7, and 2.6) found that only flows with higher PI (1.7 and 2.6) exerted detrimental effects on endothelium when no elastic deformation existed (23). Thus, we have used a PI of 1.7 to represent a high pulsatility flow condition generated by “proximal” artery stiffening. Recent studies support the strong coupling of increased upstream stiffness and downstream flow pulsatility in the highly perfused organs (1, 31).

Immunofluorescent staining for SMA and SM-MHC. After 24 h of culture, SMC in collagen were fixed with methanol at −20°C for 10 min. To obtain high-contrast images, collagen matrix with SMCs was sectioned in 5-μm slices. Collagen gels were embedded in Optimal Cutting Temperature compound (Tissue-Tech, Miami, FL) and frozen.
Frozen blocks were cut with a cryostat to obtain 5-μm sections. Following cryostat sectioning, immunofluorescent staining was performed for SM-MHC or SMA. All slides were blocked with 3% bovine serum albumin in PBS for 30 min. The primary antibody, mouse anti-SMA or mouse anti-MHC, was diluted at 1:100 in PBS and added to the slides overnight. The slides were washed before they were incubated with the secondary antibody, anti-mouse FITC IgG, which was also diluted at 1:100 in PBS. Finally, the slides were fixed with DAPI nuclear counterstain. The antibodies and DAPI stain were all obtained from Sigma.

The intensity of the fluorescent images was measured to determine differences in expression of SMA and SM-MHC caused by the different experimental conditions. Sections cut by the cryostat were viewed under an upright fluorescent microscope. Images were captured with a fixed exposure time that was held constant for all samples and then imported into Image J software (National Institutes of Health). To evaluate fluorescent intensity, cells were manually traced around the perimeter; with the use of Image J, the average fluorescent intensity for each image was calculated. The fluorescent intensity data were averaged from 10 representative cells from each experiment; four independent experiments were performed for each experimental condition, and thus 40 cells were quantified for the fluorescent intensity. The data were compared to determine the differences among the conditions. Cell size was also measured in a similar fashion. The average cell size for each experimental condition was determined.

Immunohistochemical staining for proliferating cell nuclear antigen. Sectioned slides were used for determining the status of cell proliferation. The method of immunohistochemical staining of proliferating cell nuclear antigen (PCNA) was used. PCNA levels are elevated in the S, G2, and M phases of cell mitosis and are often used as a marker for proliferation. Staining followed the given protocol by the PCNA kit provider (Invitrogen). Briefly, cells were fixed with acetone at 20°C for 10 min. Following fixation, cells were blocked for 10 min. Next, the primary antibody, a biotinylated PCNA monoclonal antibody (clone PC10), was applied to the slide for 1 h. This is followed by streptavidin-peroxidase and DAB chromogen applied for 10 and 5 min, respectively. The final step was to add hematoxylin for 2 min, followed by slide mounting. The cells were imaged with an upright microscope.
light microscope. A brightly red stained nucleus indicated proliferating cells, whereas an unstained nucleus represented nonproliferating cells. Thus the percentage of proliferating cells vs. nonproliferating cell could be determined.

**Western blot analysis.** Cells types were easily separated, since SMCs were embedded in the collagen matrix, and ECs were removed with the porous membrane. To retrieve SMC proteins, SMCs were removed from the collagen matrix by the addition of 20 mg/ml of collagenase solution (Sigma) for 30 min. Cells were spun down at 1,500 rpm; the pellet was then lysed with 75 μl of RIPA lysis buffer with 1 μl of protein inhibitor and then centrifuged at 14,000 rpm for 20 min at 4°C. To retrieve EC proteins, ECs on the porous membranes were gently scraped off the membrane, lysed with RIPA lysis buffer, and then centrifuged at 14,000 rpm for 20 min at 4°C. Supernatant was then transferred to a clean tube. Protein concentrations were found using a standard curve of BSA. Twenty micrograms of protein from each sample were separately loaded and subjected to gel electrophoresis. Following electrophoresis, the SMC protein was transferred to a nitrocellulose membrane. The membrane was then incubated with polyclonal mouse SMA antibody (1:500 dilution) or monoclonal mouse SM-MHC antibody (1:100 dilution) overnight. For analyzing EC proteins, the membrane was blot with monoclonal endothelial nitric oxide synthase (eNOS) antibody (1:200 dilution; Santa Cruz Biotech, Santa Cruz, CA), monoclonal CD143 or ACE antibody (1:50 dilution; AbD Serotec, Raleigh, NC), or TGF-β mouse anti-bovine monoclonal antibody (1:100 dilution; LifeSpan Biosciences, Seattle, WA) overnight. The next day, the membrane was incubated with anti-mouse IgG (1:4,000 dilution; Amersham Biosciences, Piscataway, NJ). Following the secondary antibody the membrane was incubated with electrochemiluminescence reagent (ECL; Amersham Biosciences) and finally exposed to hyperfilm (Amersham Biosciences) for visualization of protein expression. GAPDH was used as a reference protein to ensure equal loading of samples.

**Polymerase chain reaction.** After 24 h of experimental conditions, ECs on the porous membranes were washed with PBS. ECs were gently scraped off the membrane and placed in RNAlater to prevent the degradation of RNA. Cells were spun down at 2,000 rpm for 5 min; supernatant was removed leaving only the cell pellet. RNA was isolated from cell pellets using column purification and the RNAqueous-4PCR kit (Ambion). The lysis solution (300 μl) was used to dissolve the cell membranes. The obtained mRNA was purified, and the mRNA concentration was subsequently measured. cDNA was then synthesized using 500 ng of mRNA using the Bio-Rad iScript cDNA synthesis kit. Real-time quantitative RT-PCR primers were designed using Primer 3 software to target vasoconstrictors [endothelin (ET)-1 and angiotensin-converting enzyme (ACE)], vasodilators (eNOS), and growth factors [transforming growth factor (TGF)-β] (Table 1). The SYBR power 2× mix and iCycler iQ real-time PCR detection system (Rad MyiQ Real-Time PCR System; Bio-Rad, Hercules, CA) were used for detecting real-time quantitative PCR products from 500 ng of reverse-transcribed cDNA. The PCR thermal profile consisted of 94°C for 7 min followed by 42 cycles of 94°C for 45 s, 50°C for 45 s, and 77°C for 1 min and finally 1 cycle of 72°C for 7 min. Genes were normalized to the housekeeping gene hypoxanthine-xanthine phosphoribosyl transferase, and fold change relative to static ECs was calculated using the ΔΔCt method.

**Drug treatment with inhibitors.** To study the roles of ACE and ET-1 in mediating EC-SMC signaling under high pulsatility flow, ACE inhibitor and ET-1 receptor inhibitor were, respectively, added to the flow media in EC-SMC coculture under the high pulsatility flow. The ACE inhibitor ramiprilat was purchased from Sigma-Aldrich and used at a concentration of 0.5 μM. The ET-1 receptor inhibitor bosentan was purchased from MP Biomedical (Solon, OH) and used at a concentration of 10 μM. Bradykinin, used to stimulate nitric oxide (NO) and prostacyclin (PGI2) release, was purchased from Pierce (Rockford, IL) and used at a concentration of 1 μM. The drug concentrations were determined according to previous studies and our preliminary tests with several concentrations (14, 26). The flow culture was maintained for 24 h. Subsequently, Western blot analysis was performed on the SMCs to evaluate their expression of SMA and SM-MHC.

**Statistical analysis.** Statistical analysis was performed on the quantitative results of genetic analysis using two-way ANOVA. A significance level of P < 0.05 was used. Three or four samples for each flow condition and coculture setup were examined; statistical analysis was done to quantify the variation between these sample groups.

### RESULTS

**High pulsatility flow increases SMC expression of SMC contractile protein expression and size in the presence of EC.** Using our model pulmonary circulation (a pulse flow system connected to a vascular mimetic coculture), we found that high pulsatility flow increased the expression of the contractile protein markers SMA and SM-MHC in SMCs. Immunofluorescence and Western blot analyses showed that high pulsatility flow significantly (P < 0.05) upregulated SMA and SM-MHC protein expression in SMCs cocultured with ECs compared with either static or steady flow conditions (Fig. 2). High pulsatility flow (E/S HP) also significantly increased the SMC size compared with the static (E/S ST) and steady flow (E/S SS) under the same coculture conditions (Fig. 3).

To determine whether the increases in contractile protein expression and size of SMCs were dependent on ECs, SMC responses under similar flow conditions were studied in the absence of adjacent ECs. For this study, the porous membrane was not seeded with EC but was still used to separate SMC from directly interfacing with the flow while allowing transmural flow through the pores. Under these conditions, both SMA and SM-MHC protein expression decreased in steady and high-pulsatile flow conditions compared with the static condition (Fig. 4). Average cell sizes were not significantly different between static, steady flow, and high-pulsatile flow conditions (Fig. 5).

**High pulsatility flow does not stimulate proliferation of quiescent SMCs.** We next sought to determine whether high-pulsatile flow exerted effects on SMC proliferation. When comparing PCNA expression by SMC in the static, steady flow, and high-pulsatile flow conditions, we found no statistically significant increases in the percentage of proliferating cells under high-pulsatile flow in the presence or absence of ECs (Fig. 6) compared with the static and steady flow conditions.

**High pulsatility flow alters EC production of vasoactive and growth-regulating cytokines.** It is known that ECs are capable of regulating SMC phenotype and function through secretion of vasoactive substances such as eNOS, ACE, and ET-1 (8,

### Table 1. Primer sequence of bovine genes for real-time PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tbody>
<tr>
<td>TGF-β1</td>
<td>ACCCGGCTGCTTAATGTTGGA</td>
<td>GAGCAAACAGGTTGCGGCA</td>
</tr>
<tr>
<td>ET-1</td>
<td>GTTCGAGCTCTGCTGGCAC</td>
<td>AAATCATTCGACAGGCGAG</td>
</tr>
<tr>
<td>ACE</td>
<td>GTTCGATCATCCTCATTCTT</td>
<td>GGTGGAGTGTGCAAAGTA</td>
</tr>
<tr>
<td>eNOS</td>
<td>GATCGACGAGCTATCAGGA</td>
<td>GGAACGGGTAGACGATACG</td>
</tr>
</tbody>
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TGF-β1, transforming growth factor-β1; ET-1, endothelin-1; ACE, angiotensin-converting enzyme; eNOS, endothelial nitric oxide synthase.
Fig. 2. High pulsatility flow increased SMC expression of contractile proteins in the presence of ECs. 

A: representative images of the SMCs stained with immunofluorescence for smooth muscle α-actin (SMA) and smooth muscle (SM)-myosin heavy chain (MHC), showing the effects of flow cocultures on SMCs.

B: comparisons of SMA fluorescent intensities under static, steady, and high pulsatility flow coculture conditions. EC/SMC high pulsatility flow (E/S HP) showed increased SMA intensity. *P < 0.05 compared with EC/SMC static (E/S ST). *P < 0.05 compared with EC/SMC steady (E/S SS).

C: comparisons of SM-MHC fluorescent intensities under static, steady, and high pulsatility flow coculture conditions.

D: Western blot results showing similar changes of contractile proteins, SMA, and SM-MHC in SMC under static, steady, and high pulsatility flow cocultures.
we thus sought to evaluate the effect of high pulsatility flow on their expression in ECs. We examined ECs from three experimen-
tial conditions as follows: EC/SMC in a static coculture (E/S ST), EC/SMC coculture subjected to steady flow (E/S SS), and EC/SMC coculture subjected to high pulsatility flow (E/S HP). We found that, in the vascular mimetic coculture model, high pulsatility flow decreased eNOS mRNA expression by >50-fold compared with both control and steady flow (Fig. 7A). In contrast, the expression of the vasoconstric-
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TGF-β1 mRNA expression in EC was also evaluated because of its demonstrated effects on SMC contractile protein expression and size (5). High pulsatility flow upregulated TGF-β1 mRNA expression by >70-fold compared with the static EC/SMC coculture (E/S ST). Also, mRNA expression for TGF-β1 was significantly increased in high pulsatility flow compared with the steady flow condition (Fig. 8). Protein analysis of eNOS, ACE, and TGF-β1 expression in EC was consistent with their mRNA expressions (Fig. 9).

ACE inhibitor and ET-1 receptor inhibitor reversed high pulsatility flow-induced SMC changes in the coculture. To link the changes in gene and protein expression of ET-1 and ACE observed in ECs with the phenotypic changes found in cocul-
tured SMCs, we examined the effects of ACE inhibition (ramiprilat) and ET-1 receptor inhibition (bosentan) by adding the inhibitors to the circulating medium and then evaluating SMA and SM-MHC expressions in SMCs cocultured with ECs following 24 h of high pulsatility flow. We also examined the effects of bradykinin, which can induce NO and PGI2 production. We found that ramiprilat, bosentan, and bradykinin all significantly reduced SMC expression of SMA and SM-MHC (Figs. 10 and 11).

**DISCUSSION**

Changes in small arteries and even the microcirculation are commonly observed in pathological conditions where proximal vascular stiffening is a principal component of the disease process. In the case of pulmonary hypertension, proximal PA stiffening and distal arterial medial thickening and muscularization are two prominent pathological features (33, 45, 48). However, few studies have investigated potential pathophysi-

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ility increased) into altered paracrine signals capable of affecting SMC phenotype. These findings are consistent with other coculture studies (25, 54, 59). We previously demonstrated that SMA and SM-MHC both increased with high flow shear stress (i.e., 90 and 120 dyn/cm²) (23). Because flow magnitude and flow pulsatility are two major contributors in the calculation of hemodynamic energy (47), this study demonstrates that flow with elevated pulsatility but same mean rate increased SMC size and contractile protein expression, which supports the idea that flow pulsatility is a critical dynamic flow factor influencing vascular remodeling. The results are consistent with the finding by Eberth et al. (9) who demonstrated that increases in flow...
pulsatility caused increased arterial wall thickness and diameter. Our results are also consistent with those showing causal effects of hypertensive hemodynamics on increased SMC contraction (40, 54) and hypertrophy in pulmonary or systemic microvasculature (2, 21).

In addition to paracrine signaling from ECs to SMCs under flow, the myoendothelial gap between cocultured cells, as recently studied by Gairhe et al. (11), may also partially contribute to cell-cell interactions in this study. Herein, we used 3-D culture of SMC in collagen gel as a mimetic medial layer. Some SMCs may be in close/direct contact with EC, forming myoendothelial gap and activating TGF-β through gap junctional signaling. Perhaps consistent with the idea, our results have shown that, in the absence of ECs, high pulsatility flow had opposite effects on SMC contractile protein expression and cell size (35). One possible explanation to our observed differences in SMC contractile expression with no EC presence is the effect of transmural flow. Vascular SMC phenotype in vivo can be affected by flow through the porous elastic lamina, which contains fenestral pores with sizes ranging from 0.4 to 2.1 μm in diameter (49). Previous studies have provided equations to quantitatively determine the hydraulic permeability ($K_p$) for a 3-D culture (Eq. 1), which allows the transmural flow to reach cells, and the average transmural flow shear stress on the SMCs (Eq. 2) (4, 52):

$$K_p = \frac{\mu Q_i / A}{(\Delta P/L)}$$  \hspace{1cm} (1)

$$\tau = \frac{\mu (Q_i/A)}{\sqrt{K_p}}$$  \hspace{1cm} (2)

where $\mu$ is the fluid viscosity, $Q_i$ is the volumetric flow rate to the interstitial flow, $A$ is the cross-sectional area of the gel, $\Delta P$ is the pressure drop over the length ($L$) of the chamber, $Q_i$ defined as $Q_i = Q_{in} - Q_{out}$ is the transmural flow through the collagen gel determined by the amount of flow that diffused into this matrix along the length of the chamber, and $\tau$ is the transmural flow shear. Therefore, the transmural flow shear...
stress that SMC respond to in high pulsatility flow condition is determined to be 0.06 dyn/cm², with a direction perpendicular to the circulating flow. A similar effect of shear stress of 0.1–1 dyn/cm² on SMC contractile state was shown previously (52).

It is well known that vascular ECs sense flow shear magnitude, discriminate flow patterns, and respond to the flow by releasing molecules, including inflammatory molecules, growth factors, and vasoactive substances that affect neighboring cells (3, 25, 37). However, few studies have investigated effects of high pulsatility flow on ECs, especially those in coculture with SMC. Our results showed that high pulsatility flow influenced eNOS, ACE, ET-1, and TGF-β1 mRNA expression in EC cocultured with SMC. Decreased EC production of eNOS or release of NO, which impairs vasodilation, is a prominent characteristic of hypertensive microvasculature (37). A somewhat surprising finding here is that steady flow induced only insignificant increases in eNOS by EC in coculture with SMC compared with the static coculture condition, but high pulsatility flow significantly downregulated eNOS gene and protein expression in ECs. These results are in agreement with several recent studies using EC-SMC cocultures. Tsai et al. showed a reduction of mRNA expression of eNOS by ECs in coculture with SMC compared with ECs without SMC (53). Wang et al. and Li et al. found that flow slightly increased eNOS expression of EC mRNA by ~1.5-fold (24, 58), whereas other studies showed flow induced NO production of EC under coculture through a significant increase in eNOS phosphorylation, rather than total eNOS production (41, 52). Our results on the suppressive effect of high pulsatility flow on EC expression of eNOS seem to differ from previous studies that reported increased eNOS and NO by high pulsatile flow (16, 24, 56, 57). Significant differences exist between “high pulsatile flow” used in these previous studies and “high pulsatility flow” used here.

In previous studies, a rotary gear pump (CELLMAX) was used to generate pulsatile flows whose pulsatility and frequency directly depend on the flow rate. Therefore, the high pulsatile flow in these studies was actually “high flow” with increased mean flow rate and mean flow shear stresses accompanied by increased pressure pulsation. The flow pulsatility (PI) remained undefined, and measured pulse pressure pulsations in these studies could not directly correlate to the flow PI, since a capillary high-resistance system was used. These studies reported that, compared with low pulsatile flow (e.g., 0.3 dyn/cm²), high pulsatile flow (e.g., 10–25 dyn/cm²) increased NO by ECs. In our study, the mean flow rates in two flow conditions, high pulsatility flow (PI = 1.7) and low pulsatility or steady flow (PI = 0.2), were always the same and thus the average flow shear (12 dyn/cm²) in the physiological range; the only difference is in the PI, which is modulated by a compliance-adjustment chamber. Our previous study demonstrated that the low pulsatility flows (PI ≤ 1) were very different from high pulsatility flows in that they did not elicit endothelial dysfunction such as proinflammatory responses, or they even...

Fig. 8. The mRNA expressions for transforming growth factor (TGF)-β1 in different coculture conditions, including the static (E/S ST), steady flow (E/S SS), and high pulsatility flow (E/S HP) conditions. The mRNA levels under these conditions are normalized by the expressions from ECs without the presence of SMC in a static culture condition. *P < 0.05 compared with E/S ST. †P < 0.05 compared with E/S SS.

![Western blot results showed the protein expressions for eNOS (A), ACE (B), and TGF-β1 (C) in different coculture conditions, including the static (E/S ST), steady flow (E/S SS), and high pulsatility flow (E/S HP) flow conditions. The protein levels under these conditions are normalized by EC expressions of the housekeeping protein GAPDH. *P < 0.05 compared with E/S ST. †P < 0.05 compared with E/S SS.](http://ajplung.physiology.org/)

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could protect ECs (23). Additionally, previous studies also showed that it was p-eNOS that was increased by high pulsatile flow compared with low pulsatile flow, whereas the total eNOS was found unchanged by high and low pulsatile flow conditions (56). The results shown here demonstrate that high pulsatility flow reduced total eNOS production.

Regarding the influence of high pulsatility flow on vasoconstrictive factors, ACE expression was increased by high pulsatility flow. Consequently, inhibitors of ACE or ET-1 receptor reduced contractile protein expressions by SMCs cocultured with ECs under high pulsatility flow (E/S HP). Western blot results showed SMC expressions of SMA and SM-MHC under E/S HP were significantly reduced by inhibitors such as ramiprilat (A) and bosentan (B). Both ramiprilat (10 μM) and bosentan (10 μM) were added in the flow media. *P < 0.05 compared with E/S HP.
satillity flow, which agreed with findings showing that circulating angiotensin II contributes to SMC hypertrophy and vasoconstriction but not hyperplasia (13). Similarly, ET-1, another important vasoconstrictor released by ECs in pulmonary hypertension, which has been shown to increase SMC size and contractile proteins (33), was increased by high pulse flow. TGF-β, also increased by high pulsatility flow, is known to increase contractile protein expression in SMC as well as others (5, 39). Therefore, our results show high pulsatility flow exerts effects on ECs, which could contribute to enhanced expression of contractile proteins and vasoconstriction in pulmonary hypertension.

We acknowledge several experimental conditions/limitations that may influence the results in the study, including: 1) the manner in which a EC/SMC coculture study was performed; 2) the potential differentiation status of cultured SMCs; and 3) the lack of cyclic stress response that could accompany high pulsatility flow in vivo. To better mimic the cell conditions on the artery wall, we suspended SMCs in 3-D collagen gel and separated them from ECs with a microporous membrane allowing cell-cell communication in the vascular mimic. We attempted to induce SMC into a “normal, quiescent, nonproliferating phenotype” by depriving them of serum for 48 h before the flow experiments. However, maintenance of an entirely quiescent differentiated SMC in cell culture is difficult. Additionally, in a diseased artery, high pulsatility flow could induce increased cyclic stretch on the arterial walls; a response to stretch could contribute to increased SMC hypertrophy and hyperplasia, although stretch effects on small arteries are not as important as larger elastic arteries. While this model system was not to incorporate stretch, we previously examined its effect on SMCs. Our previous work showed that the nuclear shape, proliferation, and cytoskeletal structure of SMCs were influenced by varied cyclic stretch mechanical loading (50). Thus the addition of cyclic stretch to the high pulsatility flow system could further contribute to SMC hypertrophy and hyperplasia and should be studied in the future. In conclusion, we have demonstrated the effects of upstream stiffening on downstream SMC remodeling using a mimetic flow system with coculture, which illustrates the role that arterial stiffening may have in cardiovascular disease.

Conclusion. This study has shown that high pulsatility flow, caused by decreased upstream compliance, causes SMC hypertrophy and increased contractile protein expression. This effect is mediated by alteration in EC production of vasoactive mediators and growth factors. This study suggests that coupling of large artery compliance and small artery dysfunction might occur through hemodynamic mechanisms. Proximal arterial stiffening may significantly affect downstream arterial smooth muscle remodeling through altered pulse wave effects on ECs.

REFERENCES


