Cyclic Stretch Affects Pulmonary Endothelial Cell Control of Pulmonary Smooth Muscle Cell Growth

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Endothelial cells are subjected to mechanical forces in the form of cyclic stretch resulting from blood pulsatility. Pulmonary artery endothelial cells (PAECs) produce factors that stimulate and inhibit pulmonary artery smooth muscle cell (PASMC) growth. We hypothesized that PAECs exposed to cyclic stretch secrete proteins that inhibit PASMC growth. Media from PAECs exposed to cyclic stretch significantly inhibited PASMC growth in a time-dependent manner. Lyophilized material isolated from stretched PAEC-conditioned media significantly inhibited PASMC growth in a dose-dependent manner. This inhibition was reversed by trypsin inactivation, which is consistent with the relevant factor being a protein(s). To identify proteins that inhibited cell growth in conditioned media from stretched PAECs, we used proteomic techniques and found that thrombospondin (TSP)-1, a natural antiangiogenic factor, was up-regulated by stretch. *In vitro*, exogenous TSP-1 inhibited PASMC growth. TSP-1-blocking antibodies reversed conditioned media-induced inhibition of PASMC growth. Cyclic stretched PAECs secrete protein(s) that inhibit PASMC proliferation. TSP-1 may be, at least in part, responsible for this inhibition. The complete identification and understanding of the secreted proteome of stretched PAECs may lead to new insights into the pathophysiology of pulmonary vascular remodeling.

Keywords: pulmonary endothelium; smooth muscle cells growth; cyclic stretch; proteomics; thrombospondin-1

The endothelium, the largest organ in the human body, plays an important role in many diseases, such as acute lung injury, pulmonary and systemic hypertension, coronary heart disease, sepsis, diabetes, and stroke (1, 2). Most of the studies of endothelial cell physiology have been performed on static cultures. Under physiologic conditions, however, the endothelium is not static (3). Pulmonary artery cells are subjected to mechanical forces in the form of cyclic stretch and shear stress resulting from blood pressure and blood flow (4).

Pulmonary artery endothelial cells (PAECs) exposed to cyclic strain induce vascular endothelial cell growth factor and platelet-derived growth factor, which stimulate pulmonary artery smooth muscle cell (PASMC) growth (5, 6). PAECs also produce factors that inhibit PASMC growth, such as nitric oxide (7), prostacyclin (8), heparin-like compounds (9–11), and xanthine oxidase (12).

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CLINICAL RELEVANCE

These observations are important for understanding the pathophysiology of pulmonary vascular disease, since most studies of endothelial biology have been performed on static cultures; under physiological conditions, however, the endothelium is not motionless.

Conditioned media from confluent endothelial cells inhibits smooth muscle growth, but conditioned media from nonconfluent endothelial cells stimulate smooth muscle cell growth (13), suggesting that endothelial cells may be important in controlling smooth muscle growth in different ways depending on the physiologic conditions.

The role of PAECs in the control of smooth muscle cell growth when pulmonary arteries are exposed to increased physiologic strain has been explored using excised pulmonary arteries exposed to varying levels of stretch. The levels of stretch used in this study were chosen to mimic the amount of stretched place on the pulmonary artery with pulmonary hypertension (14). When whole pulmonary arteries were exposed to increased levels of constant stretch for 4 days, there was PASMC proliferation. Removal of the endothelial cell layer further augmented the stretch-induced PASMC growth (14), suggesting that the predominant effect of PAECs under conditions of increased stretch was inhibition of PASMC growth. As discussed elsewhere (15), a variety of intercellular communications between PAECs and PASMCs may be important in PASMC proliferation.

Extensive analyses have been done in expression profiling using various microarray platforms and large-scale cDNA sequencing projects of static and mechanically stimulated endothelial cells that have led to an increase in our understanding of endothelial physiology (16, 17). Nevertheless, there is limited information available on mechanically stressed endothelial cells regarding their gene product expression, post-translational modifications, and activities that are ultimately responsible for their function (18).

Therefore, we hypothesized that PAECs exposed to cyclic stretch secrete proteins that inhibit PASMC growth. To test this hypothesis, we measured bovine pulmonary artery smooth muscle cell (BPASMC) and human pulmonary artery smooth muscle cell (HPASMC) growth in conditioned media collected from dishes containing stretched bovine pulmonary artery endothelial cells (BPAECs) and human pulmonary artery endothelial cells (HPAECs), respectively, which were exposed to 15% cyclic strain in air. We found that PAECs undergoing cyclic stretch secreted protein(s) that inhibited PASMC growth.

We used liquid chromatography and inline tandem mass spectrometry (LC-MS/MS) to identify many of the proteins secreted by PAECs when exposed to cyclic stretch and compared these with proteins secreted by PAECs in a static state. Among the proteins up-regulated by cyclic stretch was thrombospondin

(TSP)-1, a natural antiangiogenic factor. We confirmed this finding by enzyme-linked immunosorbent assay (ELISA). We also found that exogenous TSP-1 inhibited growth of static and stretched HPASMCs. Using antibodies that blocked TSP-1 binding to its receptors CD36 and CD47, we reversed the conditioned media-induced PASC growth inhibition by media conditioned by the stretched PAECs.

MATERIALS AND METHODS

BPAECs and Smooth Muscle Cells

BPAECs and BPASMCs were isolated from bovine pulmonary arteries that were purchased as a bovine heart with pulmonary arteries attached as previously described (19). Briefly, under sterile conditions, a clamp was placed on the artery just above the cardiac muscle and below the main branches. The adventitia was removed, and the outer wall was washed with betadine and 95% ethanol. The pulmonary artery was removed with clamps at both ends. The clamps were cut free from the pulmonary arteries, and the artery was opened. The inner lining was lightly scraped to remove the endothelial cells. Small strips of the smooth muscle layer were peeled away. Scraped endothelial cells and three small strips per well were placed in a separate 6-well plate with 3 ml of RPMI-1640 (12–702 F; Cambrex Corp., East Rutherford, NJ) containing 10% fetal calf serum (FCS) and antibiotics (standard media). After 24 hours, the old media was carefully removed and replaced with fresh media. BPAECs and BPASMCs were characterized using von Willebrand (factor VIII) and α -actin antibodies.

HPAECs and Smooth Muscle Cells

HPAECs (CC-2630) were purchased from Cambrex Corp. in cryopreserved vials and grown in endothelial cell basal medium-2 (CC-3156) supplemented with (CC4176) 2% FCS, human epidermal growth factor, hydrocortisone, vascular endothelial growth factor, human fibroblast growth factor, human recombinant insulin growth factor, ascorbic acid, GA-1000 (gentamicin and amphotericin B), and heparin. HPASMCs (CC-2581) were purchased from Cambrex Corp. in cryopreserved vials and were grown in smooth muscle cell basal media (CC-3181) supplemented with (CC-3182) 5% FCS, human recombinant insulin growth factor, human fibroblast growth factor, GA 1000 (gentamicin and amphotericin B), and human epidermal growth factor.

Media for PAEC and PASC Stretch

Stretch studies for bovine cells were performed in RPMI-1640 (12–702 F). Stretch studies in human cells were performed in endothelial cell basal medium (CC-3156) and smooth muscle cell basal media (CC-3181) for endothelial and smooth muscle cells, respectively. All media was from Cambrex Corp. All stretch experiments were performed in growth factor-free and serum-free media.

Cell Stretch

PAECs were seeded at 2×10^6 cells per stretch dish for use in a mechanical strain device as previously described (20). These dishes consisted of a plastic cylinder (100-mm diameter) with a fibronectin-coated silicone elastomeric membrane attached by a silastic O-ring. Stretch dishes were prepared fresh for each set of experiments and autoclaved for 45 minutes. Fibronectin (Sigma-Aldrich Corp., St. Louis, MO) at 1.7 μ g/ml in Hanks' buffered salt solution (Cambrex Corp.) was placed in the dishes at 4°C overnight. Different protein precoatings clearly affect endothelial cell function (21). We chose fibronectin as the precoating agent for our experiments due to its ability to maintain cells attached to the silastic membrane for 6 hours at 15% strain. Other precoating agents failed to maintain cell attachment.

Cells were grown on the silastic membranes for 48 hours before stretch. On the day of stretch, the old media was removed, and the dishes were washed five times with Hanks' buffered salt solution and replenished with serum-free media.

Our stretching device (US patent no. 5,348,879, 1994) was custom built and provided by Martha Gray, Ph.D. (Massachusetts Institute of Technology, Cambridge, MA) and has been previously described (22). This device applies sinusoidal, spatial homogeneous, and isotropic biaxial strain that allows complete distension and relaxation of the membrane

within the cycle. Strain is a measure of the degree of stretch and is expressed as the percentage of the change of the cell length to resting cell length. PAECs were subjected to stretch at 60 cycles per minute with 15% strain for 6 hours in serum-free media. Parallel dishes of control cells were plated on the same fibronectin-coated membranes for 6 hours in serum-free media but not exposed to stretch. All experiments were performed in 5% CO₂ in air and balanced nitrogen at 37°C. This device allowed us to collect large amounts of conditioned media.

HPASMCs were stretched using the FX-4000 Flexercell Tension Plus (Flexcell International Corp., Hillsborough, NC), which is a computer-regulated bioreactor that applies strain through vacuum pressure. Cell cultures were regulated and deformed on flexible membrane plates. In this case, cells were grown in 6-well BioFlex plates (35 mm in diameter) in a 5% CO₂ incubator at 37°C for 48 hours on collagen/fibronectin-coated plates at a concentration of 1×10^6 cells per well. On the day of stretch, cell plates were transferred to the baseplate of the cell-stretching device and stretched with 60 cycles per minute with a sine wave, a 1:1 stretch/relaxation ratio, and a 15% maximal equibiaxial elongation. Control cells were cultured in BioFlex plates but not exposed to cell stretch (static).

Collection of Cell Supernatants and Lyophilization

At the end of the stretch experiment, the media from PAECs was removed and stored at –70°C for further analysis or dialyzed with the Spectra/Por Membrane MWCO: 3,500 from Spectrum Laboratories, Inc. (Rancho Dominguez, CA) against distilled water (8–10 changes). The dialysate was then frozen in dry ice plus methanol and/or lyophilized at 30 millitor at –70°C overnight (Freezemobile 24; Virtis Co., Gardiner, NY).

PASC Growth Assays

HPASMCs from passages 3 to 7 were plated in 6-well plates (Corning Inc., Corning, NY) at 1.25×10^4 cells per well (35 mm in diameter) in standard cell culture media. The cells were seeded sparsely to avoid confluence and contact inhibition of cell growth. Forty-eight hours after seeding, the old media was removed, and cells were growth arrested with 0.1% RPMI. Forty-eight hours after growth arrest, the media was removed, and cells were treated with test media. One 6-well plate was used for each condition. The PASCs were grown in media never exposed to PAECs, media from PAECs subjected to cyclic stretch, media from PAECs without cyclic stretch, or lyophilized powder from media collected from PAECs subjected to cyclic stretch at 37°C with 5% CO₂. All preparations were supplemented with FCS at a final concentration of 10% to stimulate PASC growth. After 72 hours of growth, the cells were harvested with trypsin/EDTA (Cambrex Corp.) and counted using a Coulter Counter ZM (Coulter Electronics, Hialeah, FL). The percent growth was calculated as (net cell growth in treated medium/net cell growth in standard medium) $\times 100$, where the net cell growth = cell growth in standard or treated medium – cell growth in growth arrest medium, as previously described (19). The percent static growth was calculated (net cell growth in media containing lyophilized conditioned media from stretched BPAECs/net cell growth in media containing lyophilized conditioned media from static BPAEC) $\times 100$.

Lactate Dehydrogenase Detection

Conditioned media from static and stretched HPASMCs was evaluate for cell death and cell lysis using a nonradioactive colorimetric quantification assay based on the measurement of lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells (Cytotoxicity Detection KitPLUS; Roche Diagnostics Corp., Indianapolis, IN).

Trypsin Treatment

Control and stretch-exposed media were treated with 100 μ g/ml of trypsin (Sigma-Aldrich Corp.) for 30 minutes at room temperature. After 30 minutes, 400 μ g/ml of trypsin inhibitor (Sigma-Aldrich Corp.) was added. HPASMCs were exposed to this trypsinized medium as described previously.

Sample Preparation and Tryptic Digestion

Control and stretch-exposed media were denatured with 0.1 M sodium bicarbonate (pH 8.0) and 6 M guanidine hydrochloride (Sigma-Aldrich

Corp.). The samples were then reduced with 10 mM dithiothreitol (DTT) for 45 minutes at 60°C and alkylated for 45 minutes in the dark with 30 mM iodoacetamide (Sigma-Aldrich Corp.). Before tryptic digestion, a buffer exchange step to 100 mM sodium bicarbonate (pH 8.0) was performed using ultrafiltration to reduce the guanidine hydrochloride concentration. For this, a Vivaspin 4 centrifugal filter with a MWCO of 10,000 Da was used and spun at $3,000 \times g$ (23); three wash cycles were performed. Proteins were digested into peptides with trypsin (Sigma-Aldrich Corp.) at a protein trypsin ratio equivalent to 50:1 for 12 hours at room temperature. After digestion, the samples were frozen at -80°C until use.

Proteomics Analyses

NanoLC-MS/MS analysis was performed on an Ettan MDLC system (GE Healthcare, Piscataway, NJ) and an LTQ mass spectrometer (ThermoFinnigan, San Jose, CA). The capillary column (150×0.075 mm) used for all LC-MS/MS analyses was from New Objective (Woburn, MA) and slurry-packed in house with $5 \mu\text{m}$, 200 Å pore size magic C18 stationary phase (Michrom Bioresources, Auburn, CA). The LC mobile phase A was 0.1% formic acid in water. The mobile phase B for LC was 0.1% formic acid in acetonitrile. Approximately $2 \mu\text{g}$ of each sample were loaded onto a Peptide Captrap column (Michrom Bioresources, Auburn, CA) at a flow rate of 10,000 nL/min in 2 minutes. The sample was eluted out of the Peptide Captrap column and directed to the capillary reverse phase column, where the peptides were separated with a gradient. The gradient was programmed with a linear increase from 2% B to 40% B in 70 minutes and from 40% B to 90% B in 5 minutes. Data-dependent ion selection was performed by using the most abundant eight ions from a full MS scan for MS/MS analysis.

Database Searching and Data Analysis

Protein/peptide identifications were obtained through a database search against human proteomic database using the SEQUEST algorithm incorporated in Bioworks software (Version 3.1; ThermoFinnigan, San Jose, CA). Search parameters used included the static modification of 57.0215 on cysteine residues; only tryptic peptides were searched. The identifications were filtered by Xcorr versus charge state: The minimum criteria were 1.9, 2.2, and 3.75 for singly, doubly, and triply charged peptide ions, respectively (24). Quantitative comparison was performed between the samples using spectral count (25) to identify proteins of interest, and then a label-free comparative quantitation method based on a peak area measurement of the parent peptide ions was performed on the proteins of interest. The quantitative comparisons were normalized by total ion current.

TSP-1 Measurement

TSP-1 concentrations were measured using a Human TSP-1 EIA Kit (cat. no. CYT168; Chemicon International, Temecula, CA) following the manufacturer's instructions. The amount of TSP-1 detected in each sample was compared with a TSP-1 standard curve, which demonstrated an inverse relationship between optical density (outer diameter 490 nm) and the cytokine concentration.

Thrombospondin Treatment of PASMCs

Human platelet-derived thrombospondin was purchased from Calbiochem (cat. no. 605225; Calbiochem, San Diego, CA). HPASMCs from passages 3 through 7 were seeded at 1.25×10^4 cells per well in 5% SmGM-2 on 6-well plates. Cells were growth arrested with 0.1% RPMI 48 hours later. Two days after growth arrest, cells were treated with 10% SmGM-2, 0.1% RPMI, or 10% SmGM-2 plus TSP-1 at 25 ng/ml, 50 ng/ml, and 150 ng/ml. Cells were harvested 72 hours later with trypsin/EDTA (Cambrex Corp.) and counted using a Coulter Counter ZM). Pre-stretched HPASMCs (*see* section on Cell Stretch) were also treated with exogenous TSP-1 using this protocol.

Blockade of Thrombospondin Receptors on PASMCs

To block TSP-1 receptors on PASMCs, two blocking antibodies were used: (1) A mouse monoclonal antibody (Ab-1) directed against the N-terminal half of the central stalk-like region of thrombospondin (MS-418-P1ABX; Lab Vision Corp., Fremont, CA) that prevents TSP-1 binding to its receptor CD36 and (2) a mouse monoclonal antibody (Ab-

3) directed against the C-terminal domain of TSP-1 (MS-420-P1ABX; Lab Vision Corp.) that blocks its binding to Integrin-Associated Protein, also known as CD47. HPASMCs were seeded as described previously and treated with Ab-1 or Ab-3 at 1.25 $\mu\text{g}/\text{ml}$ and 2.5 $\mu\text{g}/\text{ml}$ plus 10% SmGM-2. After 72 hours, cells were harvested with trypsin/EDTA and counted using a Coulter Counter ZM. Isotype negative controls were used for all experiments (Lab Vision Corp., Fremont, CA).

Statistical Analysis

Statistical analyses were performed using Statview 4.5 (Abacus Concepts, Inc., Berkeley, CA). The means, total cell counts, and percentages of cell growth were compared by ANOVA, and subsequent multiple comparisons were performed using the Scheffe test. All values were expressed as mean $\pm$ SEM. Significance was accepted at $P < 0.05$.

RESULTS

Media from BPAECs Exposed to Cyclic Stretch Inhibited PASMC Growth

BPAECs were exposed to 15% cyclic stretch for 6 hours at 60 cycles per minute in serum-free media (21% O_2 , 5% CO_2 , and balanced nitrogen). Conditioned media from static and stretched PAECs was collected and placed on static BPASMCs. Conditioned media from stretched BPAECs significantly inhibited BPASMC growth as compared with cells treated with standard media (10% RPMI) or static conditioned media (Figure 1A).

Conditioned Media from Stretched BPAECs Inhibited BPASMC-Induced Growth in a Time-Dependent Manner

To explore the effect of time in the stretch-induced BPASMC inhibition, we exposed BPAECs to 60 cycles per minute for 2, 4, and 6 hours. All the experiments were done with 15% strain. We found growth inhibition of BPASMCs that was dependent upon the length of the cyclic stretch experiment (time-dependent) of growth inhibition in comparison to cells treated with static conditioned media (Figure 1B).

Lyophilized Serum-Free Media in the Form of Powder Collected from BPAECs Exposed to Cyclic Stretch Inhibited PASMC Growth in a Dose-Dependent Manner

To determine the dose-response relationship, we lyophilized serum-free media from static BPAEC and from stretched BPAEC. BPASMC were treated with lyophilized conditioned media at 0.5 mg/ml, 2.5 mg/ml, and 4 mg/ml diluted in 10% RPMI-1640. We found a dose-response relationship in BPASMC growth inhibition when compared with growth in standard media (Figure 1C). BPASMC growth in lyophilized material from static BPAECs was not significantly different from BPASMC growth in standard media (data not shown).

Trypsin-Treated Conditioned Media from Stretched BPAECs Did Not Inhibit BPASMC Growth

To determine if the substance secreted by PAECs during cyclic stretch was a protein, serum-free media from BPAECs were treated with trypsin and trypsin inhibitor. BPASMCs were treated with media never exposed to BPAECs plus 10% FCS, trypsin-inactivated media from static BPAECs plus 10% FCS (nonstretched, control), and trypsin-inactivated media from stretched PAECs plus 10% FCS (stretch). Trypsin inactivation reversed the stretch-induced growth inhibition by the media obtained from stretched BPAECs (Figure 2).

Conditioned Media from Stretched HPAECs Inhibited HPASMC Growth

We explored these finding in human cells. Conditioned media from stretched HPAECs inhibited static HPASMCs (Figure

Figure 1. Conditioned media from bovine pulmonary artery endothelial cells (BPAECs) inhibited bovine pulmonary artery smooth muscle cell (BPASMC) growth. (A) % growth of BPASMC in static and stretch media as compared with standard media (RPMI 1640). $*P < 0.001$ versus standard media (50–52). $†P < 0.001$ versus static media. (B) % growth of BPASMCs in conditioned media from cells stretched for 2, 4, and 6 hours at 15% strain. $‡P < 0.05$ versus 2 hours. (C) % static growth is % growth of BPASMCs in lyophilized conditioned media from stretched BPAECs as compared with % growth of BPASMCs in lyophilized conditioned media from static BPAEC. Lyophilized material was concentrated and the dissolved in standard media. $§P < 0.05$ versus 0.5 mg/mL. $||P < 0.05$ versus 2 mg/mL.

3A) and stretched HPASMCs growth (Figure 3B). To rule out that that pulsatile loading of the endothelium is not inducing cell death, which could result in the release of growth inhibitory or cytotoxic agents, we measured LDH in conditioned media from static and stretched HPASMCs. We detected no difference in the concentration of LDH between these two groups.

Proteomics

To characterize the secreted proteome of stretched HPAECs, we used LC-MS. From triplicate runs and using Human

Figure 2. Percent growth of BPASMCs in conditioned media from static and stretched BPAECs and trypsin-treated conditioned media from static and stretched BPAECs as compared with percent growth of BPASMCs in standard media $*P < 0.05$ versus static media. $†P < 0.05$ versus stretched media.

Proteome Organization peptide scoring criteria ($Xcorr > 1.9$, 2.2, 3.75) (24), we identified 624 proteins in static conditioned media and 783 proteins in stretch-conditioned media (see online supplement). From these identifications, we found 36 proteins

Figure 3. Conditioned media from human pulmonary artery endothelial cells (HPAECs) inhibited human pulmonary artery smooth muscle cell (HPASMC) growth. (A) Percent growth of static HPASMCs in conditioned media from static and stretch HPAECs as compared with percent growth in standard media. (B) Percent growth of stretched HPASMCs in conditioned media from static and stretch HPAECs as compared with percent growth in standard media $*P < 0.05$ versus standard media. $†P < 0.05$ versus static media.

that seemed to be at least 3-fold up-regulated (*see* online supplement).

TSP-1 Was Up-Regulated in Conditioned Media from Stretched HPAECs

The LC/MS proteomic analysis resulted in the identification of several proteins in stretched-conditioned media that have previously been found to regulate cell growth. These included PAI-1, VIP, MMP-2, and TSP-1, of which TSP-1 was of particular interest to this study. TSP-1 was further investigated using TSP-1 peptides identified in all data sets (Figure 4A). Measurement of the ion intensities of TSP-1 peptides indicated that TSP-1 was 3.3-fold up-regulated relative to the control (Table 1). The up-regulation was confirmed by ELISA (Figure 4B).

Exogenous Human Platelet-Derived TSP-1 Inhibited PASMCM Growth

To confirm a growth inhibitory role of TSP-1 in our model, we treated static and stretched HPASMCs with exogenous human platelet-derived TSP-1. We found that TSP-1 inhibited growth in static (data not shown) and stretched HPASMCs in a dose-dependent manner (Figure 5). TSP-1 concentrations were selected according to the range of concentrations found in the conditioned media from the stretched PAECs.

TSP-1 Blocking Antibodies Reversed Stretch-Induced HPASMC Growth Inhibition in a Dose-Dependent Manner

To further explore the mechanism of TSP-1 inhibition of PASMCM, growth we used antibodies to block TSP-1 binding

TABLE 1. RELATIVE ABUNDANCE OF TSP-1 MEASURED IN PROTEOMIC ANALYSIS

Protein		Ave. Sequence Coverage	Ave. Number of Unique Peptides Identified*	Mass Spectrometry Quantitation†
Stretch	TSP-1	37.5%	36	3.3
Control	TSP-1	22.3%	23	1

Definition of abbreviation: TSP-1, thrombospondin-1.
Triplicate LC-MS runs of each sample were performed.
* Out of 107 possible tryptic peptides.
† Peak area measurements were used as described in the Methods section.

to the TSP-1 receptors CD36 and CD47. We found that both antibodies reversed the conditioned media-induced growth inhibition in a dose-dependent manner (Figure 6).

DISCUSSION

PAECs are not static; they are subjected to mechanical forces in the form of cyclic stretch from blood pressure pulsatility. Fujiwara suggested that mechanical stress keeps endothelial cells healthy (26). In a study by Birukov and colleagues, PAECs preconditioned with 5% strain were protected from thrombin-induced damage in barrier function compared with nonstretched cells (27). We have found (1) that PAECs undergoing cyclic stretch secreted protein(s) that inhibited PASMCM growth (Figures 1, 2, and 3); and (2) that TSP-1, which was up-regulated by cyclic stretch, could be responsible, at least in part, for this inhibition (Figures 4, 5, and 6).

Although the effects of cyclic mechanical stress on smooth muscle cells have been extensively studied, the effects on endothelial cells have not been investigated in detail. Ettenson and coworkers (13) demonstrated a density-dependent control on smooth muscle cell growth by endothelial cells. Conditioned media from subconfluent endothelial cells stimulated smooth muscle cell growth, whereas conditioned media from confluent endothelial cells inhibited systemic vascular smooth muscle cell growth. This effect did not result from a difference in the antiproliferative heparan sulfate component but rather from nonproteoglycans proteins that interacted with the heparan sulfate (13). In our study, we used confluent endothelial cells.

Figure 4. (A) Identification of a peptide from thrombospondin (TSP)-1 by inline tandem mass spectrometry (MS/MS) sequencing. This MS/MS spectrum shows the fragmentation pattern of one of the TSP-1 peptides used for quantitation. Sequence of peptide: K.QVTQSYWDTNPTR.A; mass of peptide: 1,596.7 Da; charge state of peptide: 2; Xcorr of peptide: 3.5. (B) TSP-1 production was up-regulated in conditioned media from stretched human PAEC. **P* < 0.05 versus static conditioned media.

Figure 5. Percent growth of stretched HPASMCs in standard media plus increasing concentrations of TSP-1. **P* < 0.05 versus standard media without TSP-1. †*P* < 0.05 versus standard media with TSP-1 at 25 mg/ml.

Figure 6. Percent growth of HPASMCs in conditioned media from static HPAECs and stretched PAECs and conditioned media from stretched HPAECs plus anti-TSP receptor antibodies. (A) Ab-1 antibody (anti-CD36). (B) Ab-3 antibody (anti-CD47). * $P < 0.05$ versus stretch media.

We chose a protocol of 15% cyclic strain at 60 cycles per minute to mimic physiologic conditions of tension and rate on the pulmonary vessels. Studies of excised canine pulmonary arteries under physiologic pressure (26 conditioned media H_2O) have shown a maximum circumferential strain of 21.5% and maximum axial strain of 36% (28). Others have reported a 5% to 6% artery wall excursion at peak systole under normal conditions, but these studies were done on ascending aorta and femoral arteries, not pulmonary arteries (29). The maximum strain achievable with our device was also 15%. Thus, the degree of strain used in our experiments approximates the amount of strain found *in vivo*. We used endothelial cells from the main pulmonary artery rather than the endothelial cells from microvasculature of the lung since endothelial cells in the microvasculature are exposed to laminar flow rather than cyclic stretch and based on the findings of Kolpakov and associates that removal of the endothelium augmented stretch-induced smooth muscle cell proliferation in proximal pulmonary arteries. However, in the largest of the main pulmonary arteries, the media is thick so that TSP-1 may be unable to migrate through to the outermost smooth muscle in the media, suggesting that *in vivo* TSP-1 release by proximal pulmonary artery endothelial cells could produce antiangiogenic effects on adjacent downstream vessels.

Several devices have been used to subject cells to cyclic strain *in vitro*. Both the devices used in this project were designed to produce uniform strain across the membrane. To collect large amounts of conditioned media from static and stretched PAECs, PAECs were stretched using a well-characterized device (22). Using permanent markers on the stretch membrane

surface, the dot motion was analyzed using a digitalizing video camera and a motion analyzer. The strain profile for this device was shown to be biaxially uniform and isotropic (radial = circumferential strain over the entire culture membrane) to within 14%. This device has been used to study the effects of cyclic stretch on vascular smooth muscle (30), fibroblasts (31), and type II-like alveolar cells (32). The second device (FX-4000 Flexercell Tension Plus) allowed us to use smaller amounts of conditioned media and a smaller number of HPASMCs. This device was used for the results in Figure 3B to decrease the amount of conditioned media that was needed to calculate the percent growth of HPASMCs. This device also delivers uniform strain across the membrane and was set to deliver 15% strain. Also, it has been used to study the effects of cyclic stretch on endothelial cells and type II epithelial cells (33, 34).

To characterize the factors responsible for the conditioned media-induced inhibition, we trypsin-digested the conditioned media obtained from PAECs undergoing cyclic stretch and found that this treatment was able to reverse the growth inhibitory effect and that cells grew similarly to those cultured on standard media (see Figure 2), showing that the substance inhibiting PSMC growth was a protein and that it was the presence of a factor(s) (*de novo* production or up-regulation) and not their absence (down-regulation) that caused this conditioned media-induced inhibition. Rosenbaum and collaborators found that hepatic endothelial cells secrete a growth inhibitor for lipocytes and hepatic fibroblasts; this growth inhibition was also blocked by trypsin inactivation (35).

The National Heart, Lung, and Blood Institute Clinical Proteomics Working Group Report encourages the use of large-scale protein identification to discover candidate disease markers that could lead the way to new hypotheses with the end result of new diagnostic methods, treatments, and cures (36). Other researchers have attempted to do large-scale proteomics analyses on human endothelial cells (18), but our group is the first to analyze proteins from static and stretched PAECs. To identify the proteins responsible for our initial observation, we performed capillary HPLC coupled to an electrospray-linear ion trap mass spectrometer. Although there are a number of proteomic approaches, nanospray LC-MS has very high sensitivity, as demonstrated by the average 650 high-confidence protein identifications made in each analysis from 2 μ g of protein. In this case, the protein of interest was not of low abundance, and the high sensitivity enabled significant sequence coverage (Table 1). The large number of identified peptides allowed for robust peak area measurements across a number of peptides. This is important as label-free quantitation was performed, and the data were normalized using the average total ion current from the triplicate analysis.

We further studied the proteins identified by mass spectrometry that have been implicated in vascular biology and cell growth regulation for validation. Since the methods of mass spectrometry we used were semi-quantitative, the findings of up-regulation or down-regulation of a particular protein needed to be confirmed by another method (37). Using ELISA for confirmation, we measure TSP-1, VIP, PAI-1, and MMP-9 production. We found that only TSP-1 was up-regulated significantly by stretch (see Figure 4B). VIP, PAI-1, and MMP-9 may also be important in PAEC control of PSMC growth, but we could not confirm their role using our model.

Others have found that TSP-1 production can be up-regulated by different types of mechanical forces. Gomez and colleagues and Bongrazio and colleagues (38, 39) reported that shear stress is capable of increasing TSP-1 secretion from endothelial cells. Furthermore, studies using laser-microdissected intrapulmonary arteries showed that TSP-1 was up-regulated 4-fold after 1 day in

an animal model of hypoxia-induced pulmonary hypertension where vessel walls are subjected to increased shear and cyclic stress due to increased pulmonary artery pressures (40). Finally, Sozo and colleagues reported a 3.5-fold increase in TSP-1 expression in response to obstruction of tracheal fluid outflow in the developing ovine lung as a result of mechanical stretch (41). Our findings confirm that TSP-1 is actively expressed by pulmonary artery endothelial cells and that it is a mechanical stress-responsive protein.

Thrombospondins are a family of five known proteins that are composed of multiple well defined structural motifs. All five members contain type 2 repeats, type 3 repeats, and a highly conserved C-terminal domain (42). TSP-1 and TSP-2 are the members of this family that have been shown to control cell proliferation (42). TSP-1 is released by platelets, macrophages, monocytes, fibroblasts, vascular smooth muscle cells, and endothelial cells (42, 43). TSP-1 has been shown to stimulate and inhibit EC and SMC growth (43, 44) and to stimulate and inhibit angiogenesis (45). These contradictory functions have been explained by the use of different experimental approaches and the ability of TSP-1 to interact with various matrix proteins and cell-surface receptors (46). However, in most circumstances, TSP-1 is antiangiogenic (45).

To confirm the possible role of TSP-1 in the growth inhibition by conditioned media from stretched PAECs, we treated HPASMCs with exogenous TSP-1. We found that TSP-1 was capable of inhibiting growth in a dose-dependent manner in static and stretched HPASMCs. The doses used in these experiments were chosen according to the concentrations we found in our conditioned media (see Figure 4). Isenberg and colleagues (47) also reported that TSP-1 inhibited human aortic vascular smooth muscle cell growth but at much higher concentrations. Isenberg's experiments were done on static aortic vascular smooth muscle cells, but not on stretched pulmonary smooth muscle cells.

CD36 is thought to be the primary receptor that mediates TSP-1 antiangiogenic activity. Some recent evidence suggests that TSP-1 also blocks angiogenesis through β_1 integrins and CD47 (48, 49). To further investigate the TSP-1 involvement in our model, we blocked TSP-1 binding to CD36 and CD47 using monoclonal antibodies. We found that by blocking each antibody independently, conditioned media-induced inhibition of PASMC growth was reversed in a dose-dependent manner. These observations parallel findings by Isenberg and colleagues, indicating that CD36 and CD47 were involved in the TSP-1 inhibition of NO-stimulated vascular smooth muscle cell proliferation (49).

A limitation of our model is the large volume of HPAEC-conditioned media needed to conduct the growth inhibition studies. To overcome this problem, we used two different stretch devices, one for cyclic stretch of endothelial cells and one for smooth muscle cells. Both the devices used in this project were designed to produce uniform strain across the membrane. To collect large amounts of conditioned media from static and stretched PAECs, PAECs were stretched using a well characterized device (22) that uses 10-cm stretch plates. Using permanent markers on the stretch membrane surface, the dot motion was analyzed using a digitalizing video camera and a motion analyzer. The strain profile for this device was shown to be biaxially uniform and isotropic (radial = circumferential strain over the entire culture membrane) to within 14%. This device has been used to study the effects of cyclic stretch on vascular smooth muscle (30), fibroblasts (31), and type II-like alveolar cells (32). To reduce the amount of conditioned media needed to be produced by the first device and to allow the use of a smaller numbers of HPASMCs, a second device was used

(FX-4000 Flexercell Tension Plus) to expose HPASMCs to cyclic stretch. The second device uses 6-well stretch plates. Each well has a diameter of 35 mm. This device was used for the results in Figure 3B. This device also delivers uniform strain across the membrane and has been used to study the effects of cyclic stretch on endothelial cells and type II epithelial cells (33, 34).

In conclusion, these observations indicate that HPAECs undergoing cyclic stretch secreted one or more proteins that inhibited PASMC growth and that TSP-1, at least in part, was responsible for this growth inhibition. Since pulmonary artery cells normally are in constant motion, the effects of cell stretch must be accounted for when analyzing results of cell culture experiments. Identification of proteins secreted by cyclic stretched PAECs that are involved in controlling vascular cell growth like TSP-1 could lead to the development of new treatment modalities for pulmonary vascular disease and primary pulmonary hypertension in which PASMC proliferation is prominent.

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