Angiogenesis is induced by airway smooth muscle strain

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Hasaneen NA, Zucker S, Lin RZ, Vaday GG, Panettieri RA, Foda HD. Angiogenesis is induced by airway smooth muscle strain. Am J Physiol Lung Cell Mol Physiol 293: L1059–L1068, 2007. First published August 10, 2007; doi:10.1152/ajplung.00480.2006.—Angiogenesis is an important feature of airway remodeling in both chronic asthma and chronic obstructive pulmonary disease (COPD). Airways in those conditions are exposed to excessive mechanical strain during periods of acute exacerbations. We recently reported that mechanical strain of human airway smooth muscle (HASM) led to an increase in their proliferation and migration. Sustained growth in airway smooth muscle in vivo requires an increase in the nutritional supply to these muscles, hence angiogenesis. In this study, we examined the hypothesis that cyclic mechanical strain of HASM produces factors promoting angiogenic events in the surrounding vascular endothelial cells. Our results show: 1) a significant increase in human lung microvascular endothelial cell (HMVEC-L) proliferation, migration, and tube formation following incubation in conditioned media (CM) from HASM cells exposed to mechanical strain; 2) mechanical strain of HASM cells induced VEGF expression and release; 3) VEGF neutralizing antibodies inhibited the proliferation, migration, and tube formations of HMVEC-L induced by the strained airway smooth muscle CM; 4) mechanical strain of HASM induced a significant increase in hypoxia-inducible factor-1α (HIF-1α) mRNA and protein, a transcription factor required for VEGF gene transcription; and 5) mechanical strain of HASM induced HIF-1α/VEGF through dual phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) and ERK pathways. In conclusion, exposing HASM cells to mechanical strain induces signal transduction pathway through PI3K/Akt/mTOR and ERK pathways that lead to an increase in HIF-1α, a transcription factor required for VEGF expression. VEGF release by mechanical strain of HASM may contribute to the angiogenesis seen with repeated exacerbation of asthma and COPD.

Airway remodeling; vascular endothelial growth factor; cyclic mechanical strain; hypoxia-inducible factor-1α

Angiogenesis afflicts the lungs of many patients with severe asthma and chronic obstructive pulmonary disease (COPD). The specific features of airway remodeling seen in asthma include hypertrophy and hyperplasia of airway smooth muscle, increased mucous glands, thickening of reticular basement membrane, and proliferation of airway blood vessels (angiogenesis; Ref. 32). Although in patients with COPD (emphysema, chronic bronchitis) there is destruction of lung parenchyma accompanied by narrowing and structural alterations of small airway (24), there is relatively little known of the cellular and molecular mechanisms underlying these structural changes (32, 38).

Lungs from patients with asthma and especially those succumbing to fatal asthma demonstrate an increase in both the number and size of blood vessels in the cartilaginous airway both inside and outside the smooth muscle layer (4, 7, 10, 11, 33). Vascular changes may contribute to airway narrowing and bronchial hyperresponsiveness seen in patients with chronic asthma by supporting the increased airway smooth muscle mass characteristic of the airways in chronic asthma, thereby allowing increased airway smooth muscle force generation (29, 30). Vascular abnormalities have also been associated with COPD (46, 53). In patients with mild to moderate COPD, there is an increase in the wall area of pulmonary vessels. The thickening correlates with the decline in forced expiratory volume in 1 s (FEV1) (43). In asthma, the enhanced vasculature is seen in the medium airway, whereas in COPD, this increased vasculature is prominent in small airways (18).

VEGF is one of the most potent angiogenic factors; it stimulates endothelial cell proliferation and induces angiogenesis. There is growing evidence to support the important role of VEGF as a mediator of vascular remodeling in asthma (35) and COPD (31). Bronchial biopsies taken from subjects with asthma reveal an increase in the expression of VEGF and VEGF receptor, which was related to the increased vascularity in the bronchial mucosa (19). VEGF expression also has been reported to correlate with airway hyperresponsiveness in subjects with asthma (1, 19, 21, 26), and VEGF levels are inversely correlated to FEV1 in patients with COPD (31). Airway smooth muscles are known to express VEGF. The regulation of this VEGF expression is not completely characterized; however, recent studies have shown that cytokines like IL-1β, TNF, and TGF-β (27) and inflammatory mediators like bradykinin and PGE2 (30) increased the expression of VEGF by airway smooth muscle.

VEGF is a primary transcriptional target of hypoxia-inducible factor-1 (HIF-1) (15). HIF-1 is a heterodimeric basic helix loop transcription factor composed of HIF-1α and HIF-1β subunits (25). HIF-1α is constitutively expressed in cells, whereas HIF-1α expression is upregulated by hypoxia as well as by a variety of growth factors and oncogenes (22). HIF-1α has been reported to initiate and directly activate the transcription of the VEGF gene, not only in response to hypoxia, but also in response to mechanical stress in the myocardium (28).

Cells of the respiratory tract are subjected to normal cyclic strain during tidal respiration. In some situations such as episodes of severe asthmatic and COPD exacerbations, cells of the bronchial wall are exposed to supernormal cyclic mechan-
ical strain (40, 51). Cyclic mechanical strain has been proposed as a possible mechanism for cell activation and may participate in the pathogenesis of the airway remodeling seen in patients with chronic asthma (12). We (16) have recently reported that mechanical strain might play a role in airway remodeling by inducing the proliferation and migration of human airway smooth muscle (HASM).

To further our understanding of the effects of mechanical strain on airway remodeling, we sought in this study to: 1) examine whether cyclic mechanical strain of HASM in vitro induces evidence of angiogenesis (increased proliferation, migration, and tube formation) in lung microvascular endothelial cells; 2) determine whether this angiogenesis was due to the increased expression and release of VEGF from the airway smooth muscles; and 3) study the mechanisms leading to the upregulation of VEGF in response to mechanical strain of these airway smooth muscles.

**METHODS**

**Cell Culture**

**HASM.** HASM were obtained from transplant donors with healthy lungs in accordance with procedures approved by the University of Pennsylvania (Philadelphia, PA) Committee on Studies Involving Humans. HASM cells were isolated from trachealis muscle and cultured as previously described (14, 41). HASM cells were isolated, purified, and cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Invitrogen). Cells from five donors in passages 1-5 were used in the studies described below.

**Human lung microvascular endothelial cells.** Human lung microvascular endothelial cells (HMVEC-L) were purchased from Clonetics (San Diego, CA). HMVEC-L were isolated from the peripheral portion of human lung; these cells are most likely predominantly pulmonary microvascular endothelial cells. HMVEC-L were grown in EGM-2MV media (human endothelial cell media supplemented with 5% FBS, 0.04% hydrocortisone, 0.4% human fibroblast growth factor, 0.1% VEGF, 0.1% insulin-like growth factor, and 1% GA-1000; Clonetics). The cells were grown in a humidified atmosphere of 95% air-5% CO₂ at 37°C and passed every 5–7 days. Cells from passages 2–5 were employed in this study.

**Stimulation of HASM With Cyclical Mechanical Strain**

HASM cells were propagated in six-well plates coated with type I collagen (Bioflex collagen I culture plate; Flexcell International, Hillsborough, NC). HASM cells were exposed to 18–20% strain and nonstrain conditions using Flexercell FX-4000 Tension Plus strain (Flexcell International) as described previously (16). HASM cells were exposed to cyclic strain for 4, 8, 12, and 24 h. In these experiments, we used 18–20% elongation for cyclic strain, which is well above the expected stretch with normal breathing (37) and has been used by many investigators to mimic the increased mechanical strain on airways during periods of bronchospasm and hyperinflation (40, 47). In another group of experiments, we exposed HASM cells to mechanical strain ranging from 5% to 20% to study the dose response relationship of strain and VEGF release.

**Angiogenesis Study**

**Preparation of HASM-conditioned media.** The conditioned media (CM) was collected from HASM cells exposed to mechanical strain and nonstrain conditions for 4, 8, 12, and 24 h. CM was then centrifuged at 1,000 g for 10 min and then passed through a 0.22-μm filter and kept at −20°C until used. Some of the CM was absorbed with VEGF neutralizing antibody (R&D Systems, Minneapolis, MN) for 30 min before use with HMVEC-L.

**HMVEC-L cell proliferation study.** HMVEC-L were seeded onto six-well plates and incubated with EGM-2MV media overnight. Then, the cells were growth-arrested for 16 h in serum-free media (basal endothelial cell media; Life Technologies). The cells were then incubated for an additional 24 h with the following: 1) CM from HASM exposed to cyclic strain for 24 h or nonstrain conditions; 2) a mixture of CM from HASM exposed to cyclic strain or nonstrain conditions with 20 or 40 μg/ml VEGF neutralizing antibody added; 3) 20 ng/ml VEGF only as a positive control; and 4) serum-free media used as a negative control.

At the end of the incubation time, HMVEC-L cell proliferation was assessed using [³H]thymidine incorporation. [³H]thymidine incorporation was used to assess DNA synthesis rates. Eight hours before the end of the experiment, 1 μCi/ml [³H]thymidine was added to cells. After 8 h of incubation at 37°C, the CM was removed. The cells were washed twice with PBS at 4°C, and cold 5% trichloroacetic acid was added for 30 min to precipitate DNA. The precipitates were washed with cold water and resuspended in 0.5 ml of 1 M NaOH, and then 0.4-ml aliquots were added to 4 ml of scintillation fluid and counted in a scintillation counter (Packard, Downers Grove, IL).

**Cell viability assay.** We examined HMVEC-L cell viability to determine the toxicity of different CM used in the proliferation assay. HMVEC-L cell viability was determined by lactate dehydrogenase (LDH) cytotoxicity assay as described previously (17).

**HMVEC-L cell migration study.** CM was obtained from HASM exposed to strain and nonstrain condition as described above. HMVEC-L migration assays were performed with Transwell (Costar) 24-well tissue culture plates with polycarbonate membranes (8-μm pores). The membranes were coated with 0.2% gelatin. HMVEC-L were seeded on the upper chamber of the Transwell at 1 × 10⁵ cells in 100 μl of basal endothelial cell media (Life Technologies) containing 0.1% BSA. Cells were exposed to one of the following: 1) CM from HASM exposed to 24 h of cyclic strain or nonstrain conditions; 2) CM from HASM exposed to cyclic strain or nonstrain conditions plus 20 or 40 μg/ml VEGF neutralizing antibody; 3) DMEM serum-free media containing 20 ng/ml VEGF as a positive control; or 4) DMEM serum-free media as a negative control. The Transwells were incubated for 24 h at 37°C in a CO₂ incubator. At the end of the experiment, the filters were fixed and stained using the Hem3 staining kit (Fisher). The number of cells that migrated to the lower surface of the membrane was counted under ×400 magnification. Five high-power random fields were counted per sample. Each group was run in triplicates at minimum.

**HMVEC-L cell tube formation.** Ninety-six-well plates were coated with cytokine-depleted Matrigel, and HMVEC-L were seeded on the polymerized matrix at a density of 1 × 10⁴ cells/well. The cells were then incubated for 16 h at 37°C in 5% CO₂ with one of the following: 1) CM from HASM exposed to 24 h of cyclic strain or nonstrain conditions; 2) CM from HASM exposed to cyclic strain or nonstrain conditions with 20 or 40 μg/ml VEGF neutralizing antibody added; 3) DMEM serum-free media containing VEGF 20 ng/ml as a positive control; or 4) DMEM serum-free media as a negative control.

Following incubation, we carefully removed the medium and washed the plate with HBSS. After washing the plates with HBSS, the plates were ready for imaging using a Nikon DXM1200 digital camera and Nikon TE2000 microscope. Tube formation was assessed and quantified by determining the mean number of branching points in six-well plates and incubated with EGM-2MV media overnight. Then, the cells were growth-arrested for 16 h in serum-free media (basal endothelial cell media; Life Technologies). The cells were then incubated for an additional 24 h with the following: 1) CM from HASM exposed to cyclic strain for 24 h or nonstrain conditions; 2) a mixture of CM from HASM exposed to cyclic strain or nonstrain conditions with 20 or 40 μg/ml VEGF neutralizing antibody added; 3) 20 ng/ml VEGF only as a positive control; and 4) serum-free media used as a negative control.

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Following incubation, we carefully removed the medium and washed the plate with HBSS. After washing the plates with HBSS, the plates were ready for imaging using a Nikon DXM1200 digital camera and Nikon TE2000 microscope. Tube formation was assessed and quantified by determining the mean number of branching points in three randomly chosen fields. Each assay was performed in triplicate, and the results are the mean of four independent experiments. To eliminate the bias, tube formation was evaluated and counted by two different investigators.

**VEGF Assay**

Concentrations of VEGF in CM at different time points were determined by ELISA (R&D Systems) according to the manufacturer’s instructions. The absorbance was determined using a microplate
reader at 450 nm. The VEGF concentrations of the unknown samples were calculated using a standard curve. We used the CM from four separate experiments; each sample was run in triplicate. The results presented are the mean of four independent experiments and expressed as picogram per microgram protein. Protein concentration of each sample was measured using BCA protein assay.

**Immunoblotting**

We used immunoblotting of HASM cell lysates to study HIF-1α, total and phosphorylated ERK, Akt, and p70S6 kinase.

HASM cells were exposed to mechanical strain or nonstrain conditions for 5 min to 24 h in the presence or absence of ERK inhibitor (PD-98059), phosphatidylinositol 3 (PI3) inhibitor (LY-294002), or rapamycin (inhibitor of mammalian target of rapamycin, mTOR). Cell lysates were then obtained by incubating the cells with lysis buffer containing 0.1% SDS, 0.5% sodium deoxycholate, and 1% Nonidet P-40 in PBS containing a protease inhibitor cocktail (Sigma, St. Louis, MO) for 1 h at 4°C. Protein concentrations were determined using BCA protein assay. Protein–equalized samples (30 μg/ml) were electrophoresed through 8–16% SDS-polyacrylamide gels. Gels were then blotted onto nitrocellulose membranes. Membranes were blocked for 1 h in PBS containing 0.1% Tween 20 and 5% milk and incubated overnight at 4°C with primary antibodies: mouse anti-human HIF-1α (BD Transduction Laboratories, BD Biosciences); rabbit anti-human total ERK, total p70S6 kinase (C-18), and phosphorylated Akt (Santa Cruz Biotechnology); and mouse anti-human phosphorylated ERK and total Akt (Santa Cruz Biotechnology) and phosphorylated p70S6 kinase Thr389 (Cell Signaling). Goat anti-rabbit or sheep anti-mouse immunoglobulin horseradish peroxidase conjugate (Amersham) were used at 1:5,000 dilutions. ECL detection was performed as per manufacturer’s instructions (Amersham). Bands were identified, analyzed, and photographed using an alpha imager.

**RT-PCR for HIF-1α and VEGF**

HASM cells were exposed to cyclic strain or nonstrain conditions for 4 h, and then total RNA was isolated and extracted from HASM cells using TRI reagent (Molecular Research Center, Cincinnati, OH) per manufacturer’s instructions. RT was performed by boiling 1 μg of RNA with 1 μM oligo(dT)12–18 (Amersham) for 1 min followed by incubation for 1 h at 37°C with the reagents 1× PCR buffer II (Sigma), 2.5 mM MgCl2 (Sigma), 1 mM dNTP (Epicient, Madison, WI), 800 U/ml ribonuclease inhibitor (Sigma), and 20 units of Moloney murine leukemia virus-RT. Reactions were terminated by incubating at 95°C for 10 min. PCR was performed using 200 ng of cDNA, 1× PCR buffer II, 1.5 mM MgCl2, and 0.5 units of Tfl DNA polymerase (Epicient), and the following oligonucleotide primers were used at a concentration of 0.4 μM: HIF-1α (forward 5′-GTG AAG GCT GTG AAG TCG TCA-3′; reverse 5′-TTC CGG CGC TTC TCG TAG ATG AA-3′), VEGF (forward 5′-CGA AGT GGT GAA GTT CAT GGA TG-3′; reverse 5′-TTC TGT ATC ATG CTT TCC TGGTGA-3′), and GAPDH (forward 5′-CCCACTACCATCT-TCCAG-3′; reverse 5′-ATGACCTTGGCCCACAGC-3′). Amplification conditions for each reaction were as follows: for HIF-1α and VEGF, initial denaturation phase was 5 min at 95°C followed by an amplification phase of 45 cycles of denaturation at 95°C for 10 s, annealing at 57°C for 10 s, elongation at 72°C for 15 s; for GAPDH, 25 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, elongation at 72°C for 1 min. PCR samples were analyzed by electrophoresis on 2% agarose gels and photographed using alpha imaging.

**p70S6 Kinase Assay**

HASM cells exposed to strain or nonstrain conditions for 24 h were scraped under ice-cold PBS and pelleted by gentle centrifugation. The cells were then resuspended in ice-cold lysis buffer (25 mM HEPES, pH 7.5, 1% Triton X-100, 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each aprotinin and leupeptin). Cell lysates were centrifuged at 15,000 g for 30 min at 4°C, and the supernatants were stored at −80°C. A quantitative protein assay was performed on each sample. HASM cell extract (50 μl) was mixed with 300 μl of lysis buffer, and 5 μl of antibody to p70S6 kinase (Santa Cruz Biotechnology) was added. After 3 h on ice, 12.5 μl of packed protein A agarose beads were added to each sample, and the tubes were agitated for 1 h at 4°C. The beads were washed twice with lysis buffer and twice with S6 kinase buffer minus dithiobiotreitol. S6 kinase activity in the immunoprecipitate was then measured using the 40S ribosomal subunit as a substrate as previously described (42). The 3P incorporated into S6 was then normalized to equal amounts of the cell lysate protein. Protein concentration of HASM cell lysate was determined with BSA protein assay.

**Analysis of the Data**

All results were reported as means ± SE. ANOVA for repeated measures was used to assess differences among the conditions when multiple time points were compared. Student’s t-test for unpaired data was used to assess the difference between conditions with P < 0.05 considered to be significant.

**RESULTS**

**CM from HASM Exposed to Strain-Induced Proliferation, Migration, and Tube Formations of HMVEC-L**

To examine whether mechanical strain of HASM would stimulate angiogenesis of the surrounding blood vessels, we collected CM from HASM cells exposed to mechanical strain (strain CM) or nonstrained control cells (control CM). CM was examined for its effect on HMVEC-L cell proliferation, migration, and tube formation, all characteristic features of angiogenesis.

Our results show that treating HMVEC-L with CM from HASM cells exposed to strain for 24 h led to a 2.07-fold increase in cell proliferation as detected by thymidine incorporation (Fig. 1A). HMVEC-L treated with CM from HASM exposed to cyclic strain for 24 h showed a 2.28-fold increase in cell migration (Fig. 1B). HMVEC-L tube formation was increased 2.48-fold when HMVEC-L were incubated with CM from HASM exposed to cyclic strain for 24 h (Fig. 1C).

Treatment of HMVEC-L with CM collected from HASM exposed to cyclic strain had no effect on cell viability compared with control cells not exposed to strain; cell viability in HMVEC-L exposed to these CM was >95% as determined by the LDH cytotoxicity assay (data not shown).

**Mechanical Strain of HASM Cells Induced VEGF Expression and Release**

HASM cells have been reported to express and secrete VEGF in response to inflammatory mediators (30). In this group of experiments, we examined whether mechanical strain of HASM led to the increased expression and release of VEGF. Our results show that VEGF is released constitutively by HASM cells. Exposing HASM cells to cyclic strain caused a significant upregulation in VEGF both at the mRNA and protein level (Fig. 2, A and B). RT-PCR of RNA extracted from HASM cells for 4 h showed a marked induction of VEGF mRNA in HASM cells exposed to cyclic strain compared with
the HASM cells not exposed to strain. RT-PCR of HASM cells exposed or not exposed to mechanical strain showed three bands with molecular weights of 121, 189, and 206, consistent with constitutive expression of the VEGF121, VEGF189, and VEGF206 isoforms. VEGF121 was the most prominent band seen. We did not examine the PCR product by direct sequencing to confirm the molecular identity of our samples as our results were consistent with previously published studies using HASM cells (30). VEGF protein was increased 3.3-fold in the CM from HASM exposed to mechanical strain for 24 h compared with CM from the control cells. When HASM cells were exposed to lower levels of mechanical strain (5% and 7%), which could represent normal tidal breathing levels of strain, there was no statistically significant increase in the levels of VEGF released compared with no strain conditions (Fig. 2, A and B).

VEGF Neutralizing Antibodies Inhibit the Proliferation, Migration, and Tube Formations of HMVEC-L

To test whether the VEGF released by HASM cells in response to cyclic strain is responsible for the increased proliferation, migration, and tube formation by HMVEC-L exposed to CM from strained HASM cells, a VEGF neutralizing antibody 20 or 40 μg/ml was used to inhibit proliferation, migration, and tube formation of HMVEC-L.

Our results show that VEGF neutralizing antibodies resulted in dose-dependent inhibition of proliferation, migration, and tube formation of HMVEC-L pretreated with CM from strained and nonstrained HASM cells. VEGF neutralizing antibody at 20 μg/ml resulted in a significant (50%) reduction in the proliferation seen when HMVEC-L were exposed to CM from strained HASM cells (P < 0.05; Fig. 3A). The increased ability for cell migration by the HMVEC-L exposed to CM from strained HASM cells was similarly reduced by ~52% with the addition of the VEGF neutralizing antibody (P < 0.05; Fig. 3B). The increased tube formation of HMVEC-L when exposed to CM from strained HASM was also significantly inhibited by 52%, from 14.9 ± 1.8 tubes formed per field to 7.8 ± 1.2 tubes formed per field in the presence of the anti-VEGF neutralizing antibody. VEGF neutralizing antibodies at 20 μg/ml resulted in a reduction in the proliferation, migration, and tube formation of HMEC-L when incubated with CM from HASM not exposed to mechanical strain (this did not reach statistical significance). When VEGF neutralizing antibodies at 40 μg/ml were used, they resulted in an almost total block of CM (from both strained and nonstrained HASM)-induced proliferation, migration, and tube formation of HMVEC-L.

Mechanical Strain of HASM Cells Induced HIF-1α

To study whether the increased release of VEGF in response to mechanical strain is due to an increased expression of HIF-1α, we examined HIF-1α protein immunoblotting of the cell lysates isolated from HASM exposed to cyclic strain for 1, 2, 4, 6, 8, 12, and 24 h and from control cells not exposed to cyclic strain. Our results show that HIF-1α was increased as early as 1 h after exposure to mechanical strain and reached its maximum at 6 h (Fig. 4A).

Using RT-PCR on RNA extracted from HASM exposed to cyclic strain and nonstrain conditions for 4 h, there was also marked induction of mRNA for HIF-1α in HASM cells exposed to cyclic strain compared with the HASM cells not exposed to strain (Fig. 4B).
We next examined the possible cellular mechanisms responsible for the strain-induced HIF-1α in HASM cells. Exposing HASM cells to cyclic strain induced a time-dependent increase in the phosphorylated form of ERK, which started within 5 min of cyclic strain and remained elevated for 1 h (Fig. 5A). Activation of ERK was inhibited by the ERK inhibitor PD-98059 (Fig. 5B). Exposing HASM cells to cyclic mechanical strain increased the phosphorylation of Akt (downstream target of PI3K). Akt phosphorylation became obvious within 10 min of cyclic strain, peaked at 30 min, remained activated up to 4 h, and then declined (Fig. 6A). PI3K inhibitor LY-294002 inhibited cyclic strain-induced Akt phosphorylation (Fig. 6B). In addition to PI3K activation, cyclic strain of HASM induced phosphorylation of p70S6 kinase (mTOR effector). p70S6 kinase phosphorylation was detected at 1 h of mechanical strain as determined by p70S6 kinase assay. The p70S6 kinase increased to 3 U/mg when HASM were exposed to cyclic strain for 1 day compared with 0.1 U/mg in control cells not exposed to strain (Fig. 7A). Rapamycin, an mTOR/ ferric reducing ability/power (FRAP) inhibitor, abrogated the phosphorylation of p70S6 kinase induced by cyclic strain of HASM (Fig. 7A and B). Furthermore, PI3K inhibitor (LY-294002) inhibited strain-induced phosphorylation of p70S6 kinase, suggesting that PI3K-dependent signaling events activated by mechanical strain of HASM may play a role in p70S6 kinase activity.

Given the observation that mechanical strain of HASM induced activation of both PI3K/Akt/FRAP and p42/p44 MAPK pathway, we set out to determine which pathway is involved in strain-mediated HIF-1α and VEGF induction in HASM. HIF-1α and VEGF protein levels were measured in the HASM cells pretreated with inhibitors [LY-294002 (PI3K inhibitor), rapamycin (an inhibitor of mTOR), and PD-98059 (ERK inhibitor)] for 30 min and then exposed to conditions of strain and nonstrain in the presence or absence of these inhibitors for 3 h (Fig. 8A) and 24 h (Fig. 8B). Our results showed that pretreatment of HASM cells with LY-294002, PD-98059, and rapamycin significantly blocked the cyclic strain-induced HIF-1α and VEGF protein in HASM cell lysates and CM, respectively (Fig. 8A and B).

**DISCUSSION**

We (16) and others (48) have recently reported that airway smooth muscle cells subjected to mechanical strain increase their proliferation and ability to migrate. For airway smooth muscle to sustain their growth, they would require an increase in the number of blood vessels supplying them. Therefore, in this study, we sought to examine whether strained HASM cells influences vascular remodeling. Our results show that exposing HASM cells to cyclic mechanical strain led to increased proliferation, migration, and tube formation of HMVEC-L, which are all characteristic features of angiogenesis. Furthermore, we found that mechanical strain led to an increased release of VEGF from HASM cells and that anti-VEGF blocking antibodies significantly attenuated the endothelial angiogenic changes caused by the strained HASM. In addition, we found that mechanical strain of HASM induced the expression of HIF-1α, a transcription factor required for VEGF expres-
sion. The induction of HIF-1α and hence VEGF by cyclic mechanical strain was mediated by both Akt/PI3K/mTOR and p42/p44 MAPK pathways. Taken together, our findings implicate a role for cyclic strain of HASM in mediating angiogenesis in the surrounding vessels.

The dilatation of capillary blood vessels is a striking feature of bronchial mucosa in fatal asthma (10, 11), and lung vascular abnormalities are also associated with the development of COPD (43). Furthermore, morphometric studies have shown that the enlarged and congested mucosal blood vessels contribute to increased airway wall thickness found in asthma (45) and small airways of COPD patients (18). The mechanisms leading to this vascular remodeling in asthma and COPD are not well-elucidated. In the current study, we show that CM from HASM exposed to cyclic mechanical strain induced angiogenic changes in lung microvascular endothelial cells by increasing their ability to proliferate, migrate, and form tubes. These results point to a novel mechanism by which mechanical strain could play a role in airway remodeling seen in asthma and support the notion that cyclic mechanical strain in addition to inflammation may play a role in this remodeling (12).

VEGF is a multifunctional angiogenic regulator that stimulates endothelial cell proliferation, survival, and blood vessel formation. VEGF has been reported to be overexpressed in asthma, and its expression correlated with the increase in blood vessel number and size found in asthma (1, 19, 21, 26). In a recent study, VEGF was shown to be a mediator not only of vascular, but also of extravascular remodeling and inflammation that enhance antigen sensitization in asthma, and VEGF production was shown to be a critical event in Th2 inflammation (35). Airway smooth muscles have been reported to express VEGF. Furthermore, proinflammatory mediators, including bradykinin, PGE2, Th2 cytokines (IL-4, IL-5, and IL-13), and TGF-β, and have been reported to stimulate VEGF release from HASM (30, 52). In this study, we report that HASM cells constitutively express and release VEGF. Furthermore, proinflammatory mediators, including bradykinin, PGE2, Th2 cytokines (IL-4, IL-5, and IL-13), and TGF-β, and have been reported to stimulate VEGF release from HASM (30, 52). In this study, we report that HASM cells constitutively express and release VEGF and that exposing HASM to cyclic mechanical strain induces an increase of VEGF expression and release from these cells. These cycles of mechanical strain, we believed, mimic the type of strain that airway smooth muscles may be subjected to during periods of asthma exacerbation. Our in vitro data presented here are consistent with the results that were obtained in an in vivo model of cardiac chamber dilation and myocyte stretch (3, 34) as well as results of other in vitro experiments with mechanical stretch in vascular smooth muscle (44), cardiac myocytes (54), and retinal capillary pericytes (50). Collectively, these results underscore a crucial role for mechanical strain (stretch) in inducing VEGF expression and release.

**Fig. 3.** VEGF neutralizing antibodies inhibited the proliferation, migration, and tube formations of HMVEC-L. A: thymidine incorporation of HMVEC-L incubated with CM from HASM exposed to strain and nonstrain conditions for 1 day in the presence of either 20 or 40 μg/ml anti-VEGF neutralizing antibodies or their corresponding isotype control antibodies showed a significant decrease in DNA synthesis in HMVEC-L treated with anti-VEGF neutralizing antibodies. HMVEC-L exposed to CM from nonstrained HASM demonstrated significant decrease in DNA synthesis in the presence of 40 μg/ml anti-VEGF neutralizing antibodies. B: HMVEC-L cell migration assays demonstrated a significant decrease in HMVEC-L cell migration toward CM from HASM exposed to strain when these cells were treated with either 20 or 40 μg/ml anti-VEGF neutralizing antibodies. HMVEC-L exposed to CM from nonstrained HASM demonstrated a significant decrease in cell migration in the presence of 40 μg/ml anti-VEGF neutralizing antibodies. C: a representative photograph of tube formation of HMVEC-L incubated with CM from HASM exposed to strain and nonstrain conditions for 1 day in the presence of either anti-VEGF neutralizing antibodies or their corresponding isotype control antibodies showing that anti-VEGF neutralizing antibodies significantly inhibited the tube formation induced by CM from HASM exposed to strain. These data represent means ± SE from 4 separate experiments, each run in triplicate; *P < 0.05.
We recently reported that cyclic mechanical strain increased in the proliferation and migration of HASM cells. This increased proliferation and migration was accompanied by an increase in the release and activation of matrix metalloproteinase (MMP) -1, -2, and -3 and membrane-type 1-MMP (MT1-MMP). We further showed that this strain-induced increase in HASM proliferation and migration was attenuated by an MMP inhibitor, suggesting that it was MMP dependent. MMPs, especially MMP-2, -9, and MT1-MMP, have been reported to contribute to both physiological and pathological angiogenesis because of their critical role in the basement membrane and interstitial matrix degradation (13, 23, 39), thereby promoting cell migration or influencing the bioavailability of growth factors. MT1-MMP may function as a fibrinolytic enzyme and mediate pericellular proteolysis in angiogenesis (20) and is correlated with the activation of the α,β3 integrin, which plays a major role during angiogenesis (8). Furthermore, both MMP-9 and MT1-MMP overexpression have been reported to promote angiogenesis through increasing VEGF (2, 49, 55). The combined effect of cyclic mechanical strain on VEGF secretion as well as MMP activation in HASM cells suggests

Fig. 4. Mechanical strain of HASM cells induced hypoxia-inducible factor-1α (HIF-1α) release and expression. A: a representative immunoblot and the densitometry results of 4 separate experiments for HIF-1α in HASM cells subjected to no strain and 20% strain conditions for 1–24 h. The results show that the level of HIF-1α protein was increased as early as 1 h after mechanical strain of HASM and reaches its maximum by 6 h (⁎P < 0.01). B: a representative RT-PCR and densitometry results of 4 separate RT-PCR experiments for HIF-1α mRNA in HASM cells exposed to strain and nonstrain conditions for 4 h. The results show an increase in HIF-1α expression in HASM exposed to strain (⁎P < 0.01).
that both events may play an important role in the airway remodeling seen in asthma. Furthermore, both MMPs and VEGF have been reported to be increased in sputum of patients with asthma exacerbation (36).

HIF-1α has been reported to induce VEGF expression not only in response to hypoxia, but also in response to other factors such as hormones, growth factors, and mechanical stress. Mechanical stress of vascular smooth muscles and rat myocardium has been reported to induce HIF-1α upregulation (5, 28). Herein, we report that mechanical strain induces the expression of HIF-1α in HASM cells. Transient increases in HIF-1α gene expression caused by cyclic mechanical strain of HASM may occur in patients during periods of asthma exacerbation (36).

Both PI3K and ERK/MAPK signaling pathways regulate various cellular responses to mechanical stress. Recent studies have shown that HIF-1α expression and activity are regulated by major signal transduction pathways including those involving PI3K and ERK/MAPK pathways. These pathways may be stimulus specific or cell type specific. We investigate the mechanisms for increased VEGF and HIF-1α expression in response to mechanical strain in airway smooth muscle. We found that the activation of both PI3K and p42/p44 MAP pathways were required for the induction of HIF-1α and VEGF expression mediated by mechanical strain. We then investigated the downstream mediators of PI3K activity necessary for regulating HIF-1α and VEGF expression in HASM. We found that Akt and p70S6 kinase were two parallel pathways that mediated strain-induced VEGF and HIF-1α expression. Our results correspond to those reported in several other cell lines. HIF-1α activation in response to hypoxia, growth factor, and stretch has been reported to be mediated through PI3K/Akt/mTOR pathway (28). ERK/MAPK pathway also has been reported to induce the expression of HIF-1α and VEGF in vascular smooth muscle (5, 9). These findings suggest that activation of both PI3K and p42/p44 MAP pathways by mechanical strain may be specific pathways for the induction of HIF-1α and its target gene, VEGF, in HASM cells.

Fig. 7. A and B: mechanical strain of HASM induced phosphorylation of p70S6 kinase; inhibition with rapamycin. A: phosphorylated p70S6 (pp70S6) kinase activity assay shows a significant increase in the phosphorylation of p70S6 kinase in HASM cells after 1 day of mechanical strain compared with nonstrained cells. B: immunoblotting of cell lysates from HASM cells exposed to strain and no strain conditions in the presence or absence of rapamycin [a mammalian target of rapamycin (mTOR) inhibitor] and LY-294002 (a PI3K inhibitor) for total and phosphorylated forms of p70S6 kinase demonstrated that mechanical strain induced an increase of total and phosphorylated forms of p70S6 kinase. Rapamycin and LY-294002 abrogated the phosphorylation of p70S6 kinase induced by mechanical strain. The pp70S6 kinase activity assay data represent means ± SE from 4 separate experiments, each run in triplicate (*P < 0.05); total and phosphorylated p70S6 kinase immunoblots are representative of 4 separate experiments that yielded similar results.

Fig. 8. Mechanical strain of HASM induced HIF-1α/VEGF through dual PI3K/Akt/mTOR and ERK pathways. A: a representative immunoblot for HIF-1α in HASM cells exposed to no strain or 20% strain conditions for 3 h in the presence of LY-294002 (PI3K inhibitor), rapamycin (an inhibitor of mTOR), PD-98059 (ERK inhibitor), or vehicle showed that the treatment of HASM cells with LY-294002, PD-98059, and rapamycin completely blocked the mechanical strain-induced HIF-1α. B: VEGF, ELISA assay of the CM from HASM cells exposed to no strain and 20% strain for 24 h in the presence of LY-294002, rapamycin, PD-98059, or vehicle. Treatment of HASM cells with LY-294002, PD-98059, and rapamycin completely blocked the strain-induced VEGF release. The immunoblot is representative of 4 separate experiments that yielded similar results. ELISA data represent the means ± SE from 6 separate experiments, each run in triplicate; *P < 0.05.
In conclusion, exposing HASM cells to mechanical strain induces intracellular signaling through the PI3K/Akt/mTOR and ERK pathways leading to an increase in HIF-1α, a transcription factor required for VEGF expression. VEGF release by HASM cells is induced by 10.220.33.6 on June 20, 2017 http://ajplung.physiology.org/ Downloaded from

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