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Airway epithelial-derived factor relaxes pulmonary vascular smooth muscle

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Farah OR, Li D, McIntyre BA, Pan J, Belik J. Airway epithelial-derived factor relaxes pulmonary vascular smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 296: L115–L120, 2009. First published October 24, 2008; doi:10.1152/ajplung.90391.2008.—The factors controlling the pulmonary vascular resistance under physiological conditions are poorly understood. We have previously reported on an apparent cross talk between the airway and adjacent pulmonary arterial bed where a factor likely derived from the bronchial epithelial cells reduced the magnitude of agonist-stimulated force in the vascular smooth muscle. The main purpose of this investigation was to evaluate whether bronchial epithelial cells release a pulmonary arterial smooth muscle relaxant factor. Conditioned media from SPOC-1 or BEAS-2B, a rat- and a human-derived bronchial epithelial cell line, respectively, were utilized. This media significantly relaxed precontracted adult but not fetal pulmonary arterial muscle in an oxygen tension-dependent manner. This response was mediated via soluble guanylate cyclase, involving AKT/PI3-kinase and neuronal nitric oxide synthase. Airway epithelial cell-conditioned media increased AKT phosphorylation in pulmonary smooth muscle cells (SMC) and reduced intracellular calcium change following ATP stimulation to a significantly greater extent than observed for bronchial SMC. The present data strongly support the evidence for bronchial epithelial cells releasing a stable and soluble factor capable of inducing pulmonary arterial SMC relaxation. We speculate that under physiological conditions, the maintenance of a low pulmonary vascular resistance, postnatally, is in part modulated by the airway epithelium.

pulmonary hypertension; pulmonary vascular resistance; fetal; hypoxic pulmonary vasoconstriction response

WE HAVE SHOWN THAT THE PRESENCE of an attached bronchus significantly reduces the rat pulmonary arterial smooth muscle contraction via a mechanism that appeared to be airway epithelium dependent (3). We reasoned that the bronchial epithelium releases a factor capable of relaxing the adjacent pulmonary arterial smooth muscle and coined the term bronchial epithelium-derived relaxing factor (BrEpRF). The fact that BrEpRF activity/expression was absent in fetal pulmonary arteries and under low oxygen concentration (4) led us to suggest that this factor is released under physiological conditions and may have an important role in the regulation of the pulmonary vascular resistance.

Our previous data demonstrated that the adjacent airway tissue modulated the pulmonary arterial smooth muscle, but the cells responsible for the release of this relaxing factor and its signaling pathway was not known. The purpose of the present study was to determine whether isolated bronchial epithelial cells are capable of releasing a pulmonary arterial muscle relaxing factor in vitro and to examine its downstream targets. Based on our previous studies (3, 4), we hypothesized that these cells secrete BrEpRF, which in turn relaxes pulmonary smooth muscle via a phosphatidylinositol 3-kinase/AKT (PI3-kinase/AKT) and nitric oxide-mediated pathways.

METHODS

Animals. All animal procedures were reviewed and approved by The Hospital for Sick Children Animal Care Committee. Fetal (21 days of gestation; $n = 4$ pregnant rats, average 15 fetuses per rat) and adult (60 days or older; $n = 40$) Sprague-Dawley rats were studied. The animals were euthanized with an overdose of pentobarbital sodium (50 mg/kg), the lungs were removed en bloc, and the pulmonary arteries and/or bronchi were dissected free.

Materials. All drugs, reagents, and chemicals were obtained from Sigma Chemical (St. Louis, MO) unless otherwise specified. AKT and P-AKT antibodies were obtained from Cell Signaling Technologies (Danvers, MA). Fura 2-AM was from Calbiochem (San Diego, CA). SPOC-1 cells were obtained from Dr. Scott Randell (Univ. of North Carolina at Chapel Hill). BEAS-2B cells were purchased from American Type Culture Collection (Rockville, MD).

Cell culture. All primary cells were isolated from adult Sprague-Dawley rats. Primary bronchial and pulmonary arterial smooth muscle cells (SMC) were isolated from second- or third-generation bronchi or arteries and cultured in DMEM (Wisent Bio-products) supplemented with 10% FBS, 0.5% Fungizone, and penicillin/streptomycin. SMC identity was confirmed by immunostaining with anti-smooth muscle-specific actin (not shown). SMCs were starved overnight with 0.5% FBS before intracellular calcium measurements or cultured serum free before assessment of AKT phosphorylation. Both SPOC-1, an immortalized rat tracheal epithelial cell line, and BEAS-2B, an immortalized human bronchial epithelial cell line, were grown in supplemented BEBM media (BEGM; Cambrex BioSciences, Rockland, ME). All references to culture media alone relate to stimulation with BEGM media not exposed to airway epithelial cells. The compositions of BEBM, BEGM, and the literature-described SPOC-1 media (19) are listed in Table 1.

Harvesting of conditioned medium and buffer. SPOC-1 or BEAS-2B cells were grown to 90% confluence before harvesting media (conditioned media). For the intracellular calcium measurements, a Krebs-Henseleit phosphate buffer (conditioned buffer) was used instead as follows. The SPOC-1 cells were grown in BEGM until 90% confluent, at which point the media was aspirated and the cells were washed with physiological buffering solution and reexposed to Krebs-Henseleit phosphate buffer overnight (145 mM NaCl, 5.6 mM KCl, 1.5 mM $CaCl_2$, 1.4 mM $MgSO_4$, 10 mM Na_2HPO_4, 5 mM glucose, pH 7.4).

Pulmonary vascular smooth muscle relaxation. Pulmonary arteries were mounted in a wire myograph (DMT-USA, Sarasota, FL) as previously described (3, 4). Briefly, third- or fourth-generation intralobar pulmonary artery ring segments (average diameter of 100 μm and length of 2 mm) were studied. Tissues were bathed in 4 ml of Krebs-Henseleit buffer (115 mM NaCl, 25 mM $NaHCO_3$, 1.38 mM $NaHPO_4$, 2.51 mM KCl, 2.46 mM $MgSO_4$, 1.91 mM $CaCl_2$, and 5.56 mM dextrose) bubbled either on air-5% CO_2 (normoxia, organ bath

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Table 1. *Culture media composition*

Additives	BEBM, mM	BEGM, mM	SPOC Media, mM (or noted otherwise)
KCl	1.5	1.5	4.16
NaCl	104	104	120.61
$CaCl_2$	0.1102	0.1102	1.05
$MgSO_4$	N/A	N/A	0.407
Glucose	6.0	6.0	17.51
$MgCl_2$	1.28	1.28	0.301
$NaHCO_3$	11.9	11.9	29.02
$NaH2PO_4$	4.12	4.12	0.500
Penicillin/streptomycin	No	No	100 U/0.1 mg/ml
Bovine pituitary extract	No	Yes	1%
Hydrocortisone	No	Yes	0.1 μg/ml
Insulin	No	Yes	10 μg/ml
Gentamicin-sulfate (GA-1000)	No	Yes	No
Retinoic Acid	No	Yes	50 nM
Transferrin	No	Yes	5 μg/ml
Triiodothyronine	No	Yes	No
Epinephrine	No	Yes	No
Human epidermal growth factor	No	Yes	25 ng/ml
Cholera toxin	No	No	0.1 μg/ml
Phosphoethanolamine	No	No	50 μM
Ethanolamine	No	No	80 μM

See METHODS for culture media description. BEGM is proprietary media of which the composition is a trade secret.

P_{O_2} = 140 Torr) or nitrogen-5% CO_2 (hypoxia, organ bath P_{O_2} = 40 Torr) and maintained at 37°C. After 1 h of equilibration, the optimal resting tension of the tissue was determined by repeated stimulation with 128 mM KCl at gradually increasing concentrations until maximum active tension was reached. All subsequent force measurements were obtained at optimal resting tension, which varied between 1 and 2 mN for fetal pulmonary arteries and 4 and 5 mN for adult pulmonary arteries. Isometric force changes were digitized and recorded online (Myodaq, DMT-USA). Contractile responses were normalized to the tissue cross-sectional area as follows: (width × diameter) × 2 and expressed as mN/mm^2. To investigate the effects of conditioned medium on pulmonary arterial muscle relaxation, these vessels were precontracted with an EC_{50} of either phenylephrine, or the thromboxane A_2 analog U46619. Fetal pulmonary arterial smooth muscle does not contract in response to phenylephrine and was only studied following U46619 stimulation. Bronchi were mounted and evaluated in the same manner as pulmonary arteries; however, the airways were precontracted with 10^{-5} M acetylcholine. All chemicals were diluted in Krebs-Henseleit buffer.

Multiple repeated exposures to the conditioned media were conducted by first precontracting the vascular muscle, evaluating the agonist-induced relaxation (i.e., medium), and then washing the vessel with fresh Krebs-Henseleit buffer until resting tension was reestablished, at which point another stimulation was conducted. The rationale for multiple stimulations relates to the fact that the relaxation response increased with each exposure and was maximal at the fourth stimulation. A maximum of six stimulations was conducted per vessel due to the poor muscle viability on the seventh and subsequent stimulations.

To evaluate the roles of the AKT/PI3-kinase and NO-cGMP pathways in the conditioned media-induced SMC relaxation, ODQ (soluble guanylate cyclase inhibitor, 10^{-5} M), 7-NINA (nNOS inhibitor, 10^{-4} M), and wortmannin (PI3-kinase inhibitor, 10^{-7} M) were added to the muscle bath in the presence of the endothelial nitric oxide synthase (eNOS) inhibitor L-N^5-(1-iminomethyl)ornithine hydrochloride (L-NIO; 10^{-4} M) 10 min before inducing pulmonary arterial contraction. All inhibitors were diluted in Krebs-Henseleit buffer. All inhibitors have been shown to inhibit their respective pathways at the concentrations used (12, 22, 23).

Intracellular calcium measurements ($_iCa^{2+}$). Primary adult rat pulmonary arterial and bronchial SMC passaged 2nd through 4th were seeded on polylysine-coated microglass coverslips (VWR, Toronto, ON, Canada) and grown to ~70% confluence, at which point they were starved overnight in 0.5% FBS/DMEM. SMC were then loaded with 5 μM fura 2-AM and 0.05% Pluronic acid and dissolved in Krebs-Henseleit buffer for 40 min at 37°C. The SMC were then washed twice with Krebs-Henseleit buffer to remove excess fura 2-AM and incubated for at least 10 min in fresh Krebs-Henseleit buffer to allow hydrolysis of the AM ester. Cover slips were mounted in a TC1-SL25 open-bath chamber (BioScience Tools, San Diego, CA) and imaged on an Olympus BX51WI upright microscope using a ×10 water-dipping objective.

Control (Krebs-Henseleit buffer) and conditioned buffer SMC were stimulated with ATP (10^{-4} M). Alternating excitation wavelengths (355 ± 5/396 ± 5 nm) were provided at ~1 excitation pair per second in conjunction with a 495-nm dichroic mirror and a 510 ± 20-nm emission filter (Chroma Technology), and paired images was collected after exposure for exactly the same duration within the 10- to 15-ms range. Fluorescent ratio values for the image were determined for each region of interest (ROI). Baseline fluorescence ratios (FR) of non-stimulated cells were collected for 30 frames before addition of ATP 10^{-4} M. The percentage increase from baseline FR levels to the peak stimulated FR was calculated for each SMC ROI. SMC percentage increase in fura 2 fluorescence was then converted to actual intracellular calcium concentrations (nM). The equation of the straight line relating nM of calcium and fluorescent ratio was estimated as: nM of calcium = (−807.5554) + (779.1031) × (fluorescent ratio). The slope and intercept for this equation were derived mathematically from fura 2-specific intracellular calcium calibration curves and used to draw a line of best fit relating nM of calcium and fluorescent ratio.

AKT phosphorylation measurements. Primary adult rat pulmonary arterial SMC were seeded on culture dishes until they reached ~90% confluence and then starved overnight in serum-free DMEM before the experiment. Cells were exposed to either conditioned buffer or Krebs-Henseleit for 3 min at 37°C. The cells were then washed twice with ice-cold PBS, exposed to lysis buffer [150 mM NaCl, 50 mM HEPES, 1% Triton X-100, 10% glycerol, 1.5 mM $MgCl_2$, 1.0 mM EGTA, and protease inhibitors (100 mM NaF, 10 mM $Na_4P_2O_7$, 10 nM okadaic acid)], and gently scraped. The lysate was incubated on ice for 30 min and centrifuged at 14,000 rpm for 15 min at 4°C. The supernatant was collected for protein concentration determination by the Bradford method, and AKT phosphorylation measurement was performed by Western blotting.

Western blotting. All samples were loaded with equal concentrations of protein (30–40 μg). The protein extracts were digested with Laemmli sample buffer (250 mM Tris·HCl, pH 6.8, 8% SDS, 30% glycerol, 0.02% bromophenol blue) at 100°C for 5 min and then electrophoresed on 7.5% SDS-PAGE gels. Proteins separated on the gel were then electrotransferred to nitrocellulose membranes on ice for 1 h at a constant voltage of 100 V. The membranes were then blocked for nonspecific proteins with 5% nonfat dry milk in TBS-T (20 nM Tris·HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20) on a flat bed shaker for 1 h at room temperature. The transblots were then incubated at 4°C on a rocking platform overnight with polyclonal antibodies raised against phospho-AKT [Phospho-Akt (Ser473); Cell Signaling, New England Biolabs, Pickering, ON] 1:1,000 dilutions in TBS-T containing 5% milk. After washing with TBS-T for 30 min, the transblots were incubated with anti-rabbit IgG conjugated with horseradish peroxidase (Cayman Chemical, Ann Arbor, MI) 1:5,000 dilutions in TBS-T containing 5% milk at room temperature for 60 min. The membranes were then washed with TBS-T for 40 min at room temperature. The membranes were then incubated with ECL reagent (Amersham) and exposed to Kodak OMAT film.

To assess levels of total AKT protein, the membranes were stripped by being incubated in 0.2 M glycine-HCl, pH 2.2, 0.1% SDS, and 1% Tween 20 for 30 min at room temperature and then reblocked with 5% milk in TBS-T and incubated with anti-AKT (Cell Signaling) overnight at a dilution of 1:1,000 in TBS-T containing 5% milk. AKT was detected with anti-rabbit peroxidase-conjugated IgG (Cayman Chemical) at a dilution of 1:5,000 in TBS-T containing 5% milk at room temperature. The P-AKT levels on imaging films were quantified densitometrically and normalized with their corresponding AKT densities using the FluoroChem 8000 imaging software.

Statistical analysis. Data were evaluated by Student's *t*-test or one- or two-way ANOVA, and multiple comparisons were performed with the Tukey-Kramer test as indicated. Statistical significance was accepted if $P < 0.05$. All statistical analyses were performed with the Number Cruncher Statistical System software (Kaysville, UT). Data are presented as means ± SE.

RESULTS

Bronchial epithelial cell media relaxes pulmonary arterial smooth muscle. Precontracted adult rat pulmonary arterial smooth muscle (phenylephrine $EC_{50} = 10^{-7}$ M) was initially exposed to SPOC-1-conditioned media added to the muscle bath in concentrations progressively increased from 0.1–15% of total bath volume. A relaxant response that was maximal at a concentration of 5% of conditioned media was observed (data not shown). For all subsequent studies, this conditioned media concentration was utilized.

Figure 1 illustrates the conditioned media relaxant effect on precontracted adult pulmonary arterial smooth muscle. The conditioned media relaxant response increased with each subsequent exposure cycle (precontraction, conditioned media exposure, and wash) and was maximal at the fourth stimulation. Repeated stimulation with conditioned media up to six times did not alter the magnitude of pulmonary arterial muscle relaxation. All subsequent studies evaluating the conditioned media relaxant response were therefore measured between the fourth and sixth repeated exposure. The magnitude of conditioned media-induced relaxation was significantly greater than observed with non-conditioned media for all repeated stimulations.

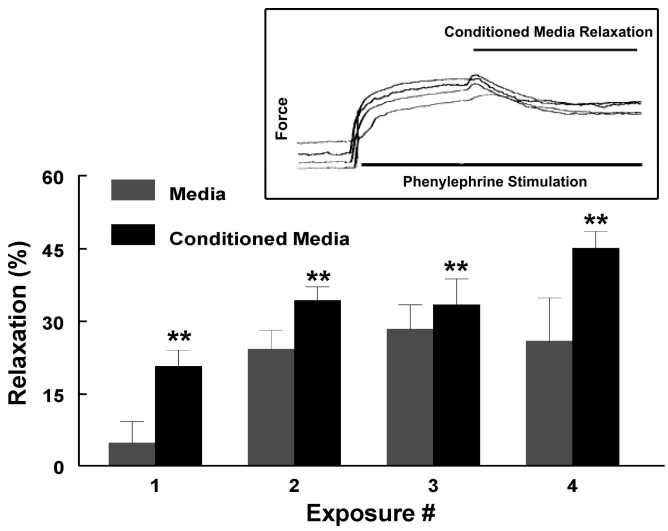

Fig. 1. SPOC-1 cell-conditioned media and BEGM (media) induced relaxation of adult rat pulmonary arterial smooth muscle precontracted with phenylephrine (10^{-7} M). Vessels were repeatedly exposed to either media up to 4 times. Conditioned media, $n = 16$; media, $n = 12$. $^{**}P < 0.01$ vs. media by 2-way ANOVA. *Inset* depicts 4 pulmonary arteries precontracted simultaneously with phenylephrine and exposed to SPOC-1-conditioned media. Note the decrease in force (relaxation) following conditioned media exposure.

The bronchial epithelial-conditioned media relaxant response was also observed in adjacent bronchi of the same generation as the pulmonary arteries. A comparison of the magnitude of conditioned media-induced relaxation (4th exposure) of pulmonary and bronchial smooth muscle is shown in Fig. 2, and it demonstrates a significantly lower response in the latter ($P < 0.05$).

Nonexposed culture media-induced relaxation. As demonstrated in Fig. 1, the commercially utilized culture media (BEGM) induces a significant relaxation response in precontracted adult pulmonary arteries. To determine the mechanism accounting for this response, we individually tested all probable constituents of this media dissolved in the basic salts plus dextrose solution (BEBM) at the approximate concentration thought to be present in BEGM. These concentrations were based on the previously published SPOC-1 cell line culture media composition (6) and were as follows: GA-1000 (gentamicin and amphotericin B) = 1 μl/ml (BEGM concentration); hydrocortisone = 0.1 μg/ml; triiodothyronine = 6.51 μg/ml; human epidermal growth factor = 25 ng/ml; and insulin = 10 μg/ml.

Phenylephrine-precontracted adult pulmonary arterial muscle relaxation to these different compounds was measured following four repeated stimulations. Insulin was the only factor that showed any response resulting in an 11.5 ± 3% relaxation ($n = 4$). We further measured the BEGM-induced relaxation of these vessels in the absence ($n = 3$) and presence ($n = 4$) of L-NIO (10^{-4} M). The relaxation decreased from 34 ± 4 to 17 ± 4% of initial phenylephrine-induced contraction ($P < 0.05$) suggesting that insulin-induced response is eNOS mediated (data not shown).

Conditioned media relaxant response is absent in the fetus and under hypoxic conditions. We evaluated the effect of bronchial epithelial cell-conditioned media on adult vs. fetal rat pulmonary arteries to determine whether the relaxation response was developmentally regulated. As shown in Fig. 3, conditioned media-induced relaxant response was absent in the fetal vessels.

Adult pulmonary arteries were also studied under hypoxic conditions to evaluate the bronchial epithelial-conditioned, media-induced relaxant response. In a muscle bath containing nitrogen and 5% CO_2 ($Po_2 = 40$ Torr), the conditioned media relaxation response was significantly reduced ($P < 0.01$) compared with air and 5% CO_2 bubbling ($Po_2 = 140$ Torr) (Fig. 4).

Last, we tested conditioned media derived from a human bronchial epithelial cell line (BEAS-2B) cultured in BEGM media. Following repeated exposures, a progressive increase in the response was observed in precontracted adult pulmonary arteries that amounted to 56 ± 5% relaxation (data not shown).

Relaxant response is soluble guanylate cyclase dependent. The conditioned media-induced relaxation of adult pulmonary arterial muscle was reduced ($P < 0.01$) in the presence of the specific eNOS inhibitor (L-NIO, 10^{-4} M; Fig. 5). Addition of the specific nNOS inhibitor (7-NINA, 10^{-4} M) and the PI3/AKT blocker (wortmannin, 10^{-7} M) to L-NIO further decreased the conditioned media-induced relaxation ($P < 0.05$;

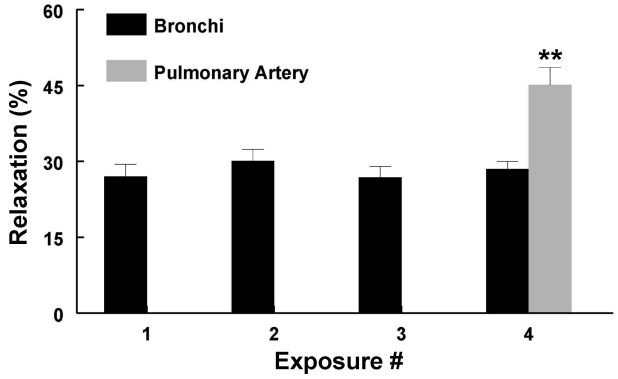

Fig. 2. SPOC-1 cell-conditioned, media-induced relaxation of adult rat bronchial ($n = 16$) and pulmonary arterial ($n = 12$) smooth muscle, precontracted with acetylcholine (10^{-5} M) 1st to 4th exposure and phenylephrine (10^{-7} M) 4th exposure, respectively; $^{**}P < 0.01$ vs. bronchi by Student's t-test.

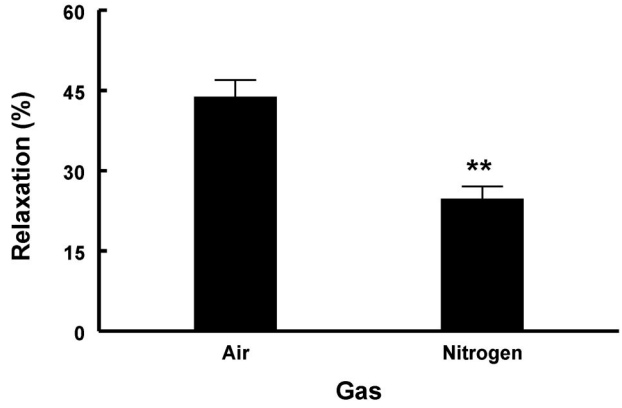

Fig. 4. Fourth exposure to SPOC-1 cell-conditioned media in adult rat pulmonary arterial smooth muscle precontracted with phenylephrine (10^{-7} M), studied in either air + 5% CO_2 (air; $n = 50$) or nitrogen + 5% CO_2 (nitrogen; $n = 7$) bubbled in Krebs-Henseleit. $^{**}P < 0.01$ compared with air group by Student's t-test.

Fig. 5). Last, the soluble guanylate cyclase inhibitor ODQ (10^{-5} M) completely abolished the relaxant response resulting in conditioned media-induced contraction (Fig. 5).

Conditioned media reduces agonist-induced $_i Ca^{2+}$ concentrations and induces AKT phosphorylation in primary pulmonary arterial SMC. To evaluate the direct effect of conditioned media on the pulmonary arterial smooth muscle, primary cells were cultured, and the $_i Ca^{2+}$ response to ATP stimulation was measured. Passaged second-to-fourth adult rat SMC response to ATP was significantly reduced in the presence of conditioned media ($P < 0.01$; Fig. 6), and the magnitude of response was significantly greater ($P < 0.05$) than similar passage number primary bronchial SMC (Fig. 7).

Last, to further confirm that the relaxant factor released by SPOC-1 cells acts directly on the pulmonary arterial muscle via the PI3-kinase/AKT pathway, we studied its effect on primary adult rat pulmonary arterial SMC. When exposed to SPOC-1-conditioned buffer for 5 min, the pulmonary arterial cells exhibited a significant increase in Akt phosphorylation ($P < 0.05$; Fig. 8).

DISCUSSION

We showed that a rat airway-derived lung epithelial cell line actively produces a stable and soluble factor capable of relaxing pulmonary arterial smooth muscle. This relaxant effect is developmentally regulated, oxygen sensitive, and of greater magnitude of response in the pulmonary arterial compared with bronchial smooth muscle. The signal transduction pathway responsible for this airway epithelium-derived arterial muscle relaxation is soluble guanylate cyclase and Akt phosphorylation dependent.

The factors accounting for the physiological regulation of the pulmonary vascular resistance are not well understood. Under normal conditions, the pulmonary vascular resistance is low, allowing for adequate blood flow through the lungs to achieve optimal gas exchange. Only two conditions are physiologically associated with a transiently high pulmonary vascular resistance: prenatal life and reduced alveolar ventilation. The latter is characterized as the hypoxic pulmonary vasoconstriction response. The mechanism responsible for the maintenance of a high vascular resistance under either one of these conditions remains poorly understood.

We have previously reported on the existence of a cross talk between the pulmonary arterial smooth muscle and the adja-

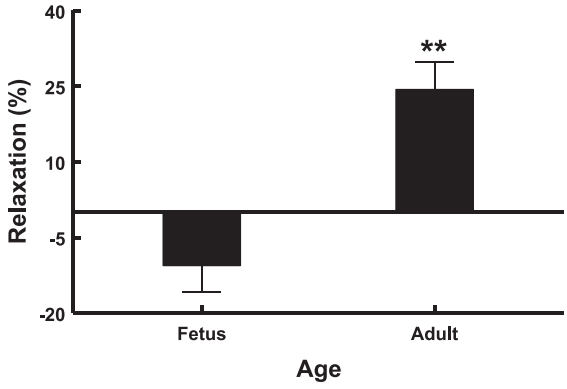

Fig. 3. Fourth exposure to SPOC-1 cell-conditioned media in fetal ($n = 4$) and adult ($n = 3$) pulmonary arteries precontracted with U46619 (fetus = 10^{-6} M; adult = 10^{-8} M). $^{**}P < 0.01$ compared with fetal arteries by Student's t-test.

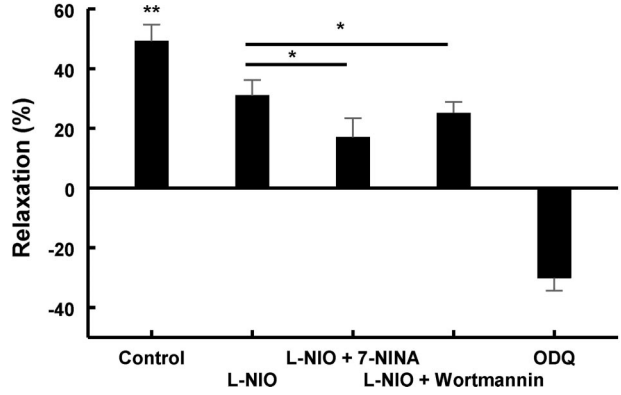

Fig. 5. SPOC-1 cell-conditioned media-induced relaxation of adult rat pulmonary arterial smooth muscle precontracted with phenylephrine (10^{-7} M), 4th-6th exposures, in the absence (control; $n = 23$) and presence of the eNOS inhibitor L-NIO (10^{-4} M; $n = 16$) alone or in combination with the nNOS inhibitor 7-NINA (10^{-4} M; $n = 4$), the PI3-kinase/AKT inhibitor wortmannin (10^{-7} M; $n = 4$), or the soluble guanylate cyclase inhibitor ODQ (10^{-5} M; $n = 4$). $^{*}P < 0.05$; $^{**}P < 0.01$ compared with all other groups by 1-way ANOVA and Tukey-Kramer for multiple comparisons.

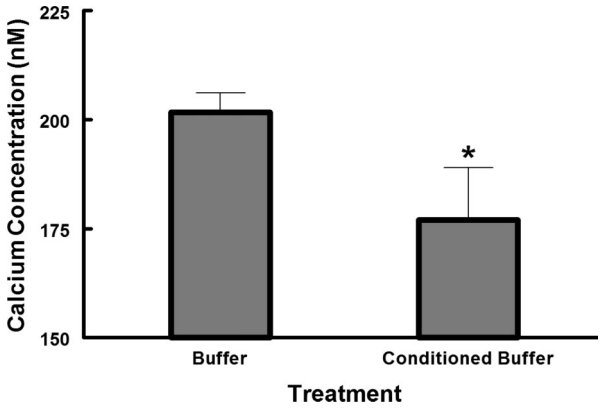

Fig. 6. Effect of SPOC-1-conditioned buffer on primary pulmonary arterial smooth muscle cell $_{i}Ca^{2+}$ stimulated with ATP (10^{-4} M). Conditioned buffer significantly decreased the ATP-induced $_{i}Ca^{2+}$ change (*$P < 0.05$ vs. buffer). Buffer, $n = 234$; conditioned buffer, $n = 86$ cells.

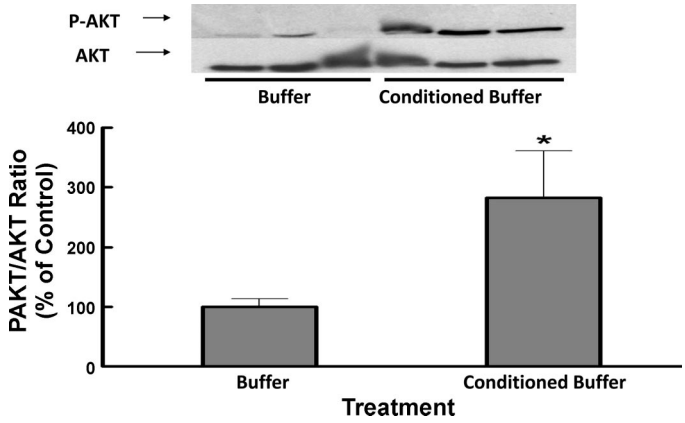

Fig. 8. Effects of SPOC-1-conditioned buffer on AKT phosphorylation in primary pulmonary arterial smooth muscle cells. In contrast to buffer alone, conditioned buffer directly stimulated AKT phosphorylation in these cells (*$P < 0.05$; $n = 12$ for each group). A representative Western blot is illustrated.

cent airway (3, 4). Data obtained from pulmonary arteries studied in the absence and presence of the adjacent airway suggested that the bronchial epithelium secreted a soluble factor capable of relaxing the pulmonary arterial smooth muscle (3, 4). These experiments, however, did not allow for an objective determination of the role of the bronchial epithelium and the signal transduction pathway responsible for airway-mediated pulmonary arterial muscle relaxation.

To overcome these limitations and confirm the role of the airway in the regulation of pulmonary vascular tone, we sought out to evaluate the in vitro epithelial cell release/secretion of a relaxant factor. Immortalized adult rat airway epithelial cells from a well-characterized cell line known as SPOC-1 were utilized. These cells originated from the rat trachea (6) but have been shown to produce mucin and have a phenotype otherwise similar to bronchial epithelial cells (1, 5, 7, 8, 15, 21). They initially form a stratified squamous epithelium, which subsequently gives rise to a more cuboidal, pseudostratified glandular epithelium that is capable of reacting with antibodies for keratins 13, 14, and 19 (6).

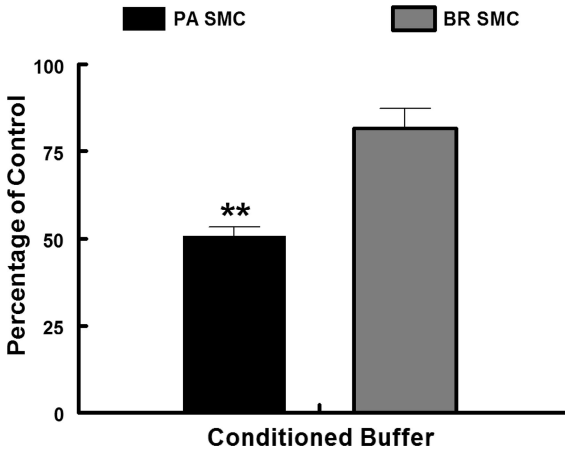

Fig. 7. Comparison of SPOC-1-conditioned buffer effect on primary pulmonary arterial (PA SMC) and bronchial (BR SMC) smooth muscle cell $_{i}Ca^{2+}$ stimulated with ATP (10^{-4} M), as a percentage of buffer alone. The magnitude of $_{i}Ca^{2+}$ reduction following exposure to conditioned buffer was greatest in PA SMC. **$P < 0.01$ vs. BR SMC. BR SMC, $n = 86$ cells; PA SMC, $n = 108$ cells.

The epithelial cell relaxant response documented in this study mimics the results previously reported for pulmonary arteries studied with and without the adjacent bronchus. Namely, the relaxant response was present in pulmonary arteries obtained from postnatal (newborn), but not fetal, rats, and it was absent under hypoxic conditions (4).

Repeated conditioned media exposures were required to obtain the maximal pulmonary arterial relaxant response at the fourth stimulation. The reasons for repeated exposures to achieve maximal response are unclear. We did not observe an incremental response to the force-reducing effect of the attached bronchus on the pulmonary arterial muscle in the previously reported studies evaluating pulmonary arteries and bronchi together (3). Assuming that in vitro repeated stimulations are required to reach the equivalent in vivo effect, we chose to compare the tissue-specific maximal response. This maximal response is more likely to be representative of the in vivo behavior and for the pulmonary arteries is observed at the fourth stimulation.

In addition, we tested the conditioned media from another airway epithelial cell line, human bronchial epithelial BEAS-2B cells, which have also been shown to preserve the phenotype of the original cells (20). The BEAS-2B cells are originally derived from human airway epithelium suggesting that the BrEpRF is operative and of physiological significance in the control of human pulmonary vascular resistance.

Together, this current and our previous studies (3, 4) confirm that airway epithelial cells do indeed release a factor capable of relaxing the pulmonary arterial muscle of the newborn and adult under normoxic conditions.

The signal transduction pathway responsible for this airway epithelium-dependent relaxation was further confirmed to involve the soluble guanylate cyclase and Akt. In keeping with our previous observation of wortmannin partial abrogation of the adjacent bronchial relaxant response (3), we have now shown that conditioned media induces pulmonary arterial SMC Akt phosphorylation. Similarly, the inhibition of the soluble guanylate cyclase and nNOS with ODQ and 7-NINA, respectively, suppressed the conditioned media relaxant response, confirming that it is NO-cGMP mediated as previously suggested (3). The nature of this bronchial epithelium-derived

relaxant factor remains unknown and is under current investigation in our laboratory.

The lack of conditioned media-induced relaxant response in fetal, compared with adult, pulmonary arteries deserves comment. Fetal pulmonary arteries, when studied in vitro under normoxic conditions (air + 5% CO_2), do not relax in response to airway epithelial cell-derived relaxing factor. The lack of response in these arteries is unrelated to their inability to relax. This is based on our previously reported data indicating that U46619-precontracted fetal rat pulmonary arteries exhibit relaxation in response to the nitric oxide donor sodium nitroprusside (10). It is also unlikely that the reduced intrauterine oxygen environment rendered these arteries unresponsive to cGMP-mediated relaxation. We have previously shown that chronic hypoxia-exposed newborn rat pulmonary arteries relax in response to sodium nitroprusside (13). This evidence suggests that a developmental immaturity of certain elements in the pulmonary arteries, and not hypoxia, account for the absent conditioned media-induced relaxant response in the fetal vessels.

To determine the factors in the nonexposed commercially used culture media (BEGM) responsible for the relaxation, we tested the ones previously reported to be associated with vascular smooth muscle relaxation. These included gentamicin (11), hydrocortisone (9), triiodothyronine (14), human epidermal growth factor (18), and insulin (2, 16, 17). Only insulin induced a significant pulmonary arterial muscle relaxation, suggesting that it is the factor responsible for the cell-independent culture media effect. Others have previously reported that insulin induces vascular smooth muscle relaxation via an endothelium-dependent and likely eNOS-mediated mechanism (24), and this is operative in rat pulmonary arteries (2). L-NIO significantly reduced the non-conditioned media (BEGM)-induced relaxation, suggesting that this response is eNOS-mediated. For this reason, we chose to evaluate the role of nNOS and PI3-kinase/Akt pathways in the BrEpRF-mediated relaxation in the presence of L-NIO to reduce and/or eliminate the insulin-related effect (Fig. 5).

In summary, we have provided further evidence for the airway epithelium secretion of a soluble and stable factor capable of relaxing pulmonary arterial smooth muscle. The fact that this relaxant response is observed neither in fetal pulmonary arteries nor in the presence of hypoxia strongly suggests that this relaxant factor plays a physiologically important role in the control of pulmonary vascular tone, postnatally. Identification of this relaxant factor and its signal transduction pathway may enable newer approaches to the prevention and/or treatment of pulmonary hypertension.

GRANTS

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REFERENCES

1. **Abdullah LH, Bundy JT, Ehre C, Davis CW.** Mucin secretion and PKC isoforms in SPOC1 goblet cells: differential activation by purinergic agonist and PMA. *Am J Physiol Lung Cell Mol Physiol* 285: L149–L160, 2003.
2. **Aye M, Sheedy W, Harrison R, Thompson JS, Morice AH, Masson EA.** Pulmonary vasodilation in the rat by insulin in vitro could indicate potential hazard for inhaled insulin. *Diabetologia* 46: 1199–1202, 2003.
3. **Belik J, Pan J, Jankov RP, Tanswell AK.** A bronchial epithelium-derived factor reduces pulmonary vascular tone in the newborn rat. *J Appl Physiol* 96: 1399–1405, 2004.
4. **Belik J, Pan J, Jankov RP, Tanswell AK.** Bronchial epithelium-associated pulmonary arterial muscle relaxation in the rat is absent in the fetus and suppressed by postnatal hypoxia. *Am J Physiol Lung Cell Mol Physiol* 288: L384–L389, 2005.
5. **Davis CW.** Regulation of mucin secretion from in vitro cellular models. *Novartis Found Symp* 248: 113–125, 2002.
6. **Doherty MM, Liu J, Randell SH, Carter CA, Davis CW, Nettesheim P, Ferriola PC.** Phenotype and differentiation potential of a novel rat tracheal epithelial cell line. *Am J Respir Cell Mol Biol* 12: 385–395, 1995.
7. **Ehre C, Rossi AH, Abdullah LH, De PK, Hill S, Olsen JC, Davis CW.** Barrier role of actin filaments in regulated mucin secretion from airway goblet cells. *Am J Physiol Cell Physiol* 288: C46–C56, 2005.
8. **Ehre C, Zhu Y, Abdullah LH, Olsen J, Nakayama KI, Nakayama K, Messing RO, Davis CW.** nPKCepsilon, a P2Y2-R downstream effector in regulated mucin secretion from airway goblet cells. *Am J Physiol Cell Physiol* 293: C1445–C1454, 2007.
9. **English KM, Jones RD, Jones TH, Morice AH, Channer KS.** Gender differences in the vasomotor effects of different steroid hormones in rat pulmonary and coronary arteries. *Horm Metab Res* 33: 645–652, 2001.
10. **Fornaro E, Li D, Pan J, Belik J.** Prenatal exposure to fluoxetine induces fetal pulmonary hypertension in the rat. *Am J Respir Crit Care Med* 176: 1035–1040, 2007.
11. **Gergawy M, Vollrath B, Cook D.** The mechanism by which aminoglycoside antibiotics cause vasodilation of canine cerebral arteries. *Br J Pharmacol* 125: 1150–1157, 1998.
12. **Hershenson MB, Wylam ME, Punjabi N, Umans JG, Schumacker PT, Mitchell RW, Solway J.** Exposure of immature rats to hyperoxia increases tracheal smooth muscle stress generation in vitro. *J Appl Physiol* 76: 743–749, 1994.
13. **Jankov RP, Kantores C, Pan J, Belik J.** Contribution of xanthine oxidase-derived superoxide to chronic hypoxic pulmonary hypertension in neonatal rats. *Am J Physiol Lung Cell Mol Physiol* 294: L233–L245, 2008.
14. **Krasner LJ, Wendling WW, Cooper SC, Chen D, Hellmann SK, Eldridge CJ, McClurken JB, Jeevanandam V, Carlsson C.** Direct effects of triiodothyronine on human internal mammary artery and saphenous veins. *J Cardiothorac Vasc Anesth* 11: 463–466, 1997.
15. **Kreda SM, Okada SF, van Heusden CA, O'Neal W, Gabriel S, Abdullah L, Davis CW, Boucher RC, Lazarowski ER.** Coordinated release of nucleotides and mucin from human airway epithelial Calu-3 cells. *J Physiol* 584: 245–259, 2007.
16. **Lee JH, Ragolia L.** AKT phosphorylation is essential for insulin-induced relaxation of rat vascular smooth muscle cells. *Am J Physiol Cell Physiol* 291: C1355–C1365, 2006.
17. **Myung SC, Keum EM, Park SY, Lee MY, Kim SC.** Vasomotor action of insulin on the rabbit normal cavernous smooth muscle. *Eur J Pharmacol* 536: 142–147, 2006.
18. **Petitclerc E, Poubelle PE, Marceau F.** Epidermal growth factor-induced rapid relaxation of the isolated rabbit mesenteric artery. *Eur J Pharmacol* 259: 91–94, 1994.
19. **Randell SH, Liu JY, Ferriola PC, Kaartinen L, Doherty MM, Davis CW, Nettesheim P.** Mucin production by SPOC1 cells–an immortalized rat tracheal epithelial cell line. *Am J Respir Cell Mol Biol* 14: 146–154, 1996.
20. **Reddel RR, Salghetti SE, Willey JC, Ohnuki Y, Ke Y, Gerwin BI, Lechner JF, Harris CC.** Development of tumorigenicity in simian virus 40-immortalized human bronchial epithelial cell lines. *Cancer Res* 53: 985–991, 1993.
21. **Rossi AH, Sears PR, Davis CW.** Ca^{2+} dependency of "Ca^{2+}-independent" exocytosis in SPOC1 airway goblet cells. *J Physiol* 559: 555–565, 2004.
22. **Secondo A, Sirabella R, Formisano L, D'Alessio A, Castaldo P, Amoroso S, Ingleton P, Di Renzo G, Annunziato L.** Involvement of PI3-K, mitogen-activated protein kinase and protein kinase B in the up-regulation of the expression of nNOSalpha and nNOSbeta splicing variants induced by PRL-receptor activation in GH3 cells. *J Neurochem* 84: 1367–1377, 2003.
23. **Tsui DY, Gambino A, Wanstall JC.** *S*-nitrosocaptopril: in vitro characterization of pulmonary vascular effects in rats. *Br J Pharmacol* 138: 855–864, 2003.
24. **Verma S, Arikawa E, Yao L, Laher I, McNeill JH.** Insulin-induced vasodilation is dependent on tetrahydrobiopterin synthesis. *Metabolism* 47: 1037–1039, 1998.