Actin polymerization contributes to enhanced pulmonary vasoconstrictor reactivity after chronic hypoxia

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INTRODUCTION

The adult pulmonary circulation consists of a low-pressure, low-resistance vasculature that normally exhibits little to no basal myogenic tone (21). However, chronic hypoxia (CH), which is associated with prolonged high-altitude exposure and chronic obstructive pulmonary diseases, increases pulmonary vascular resistance and leads to pulmonary hypertension (44). The resulting increase in afterload on the right heart causes right ventricular hypertrophy and can ultimately lead to right heart failure and death. Pulmonary artery narrowing attributable to acute hypoxic pulmonary vasoconstriction is widely thought to significantly contribute to elevated vascular resistance (45). However, additional vasoconstrictor influences, including elevated basal vascular smooth muscle (VSM) tone and enhanced sensitivity to vasoconstrictor stimuli such as endothelin-1 (ET-1), are thought to play an important role in CH-induced pulmonary hypertension (17, 29, 30, 43).

VSM contraction is largely determined by the phosphorylation state of the regulatory protein myosin light chain (MLC), which is regulated by the balance of activities of MLC kinase (MLCK) and MLC phosphatase (MLCP). Like in the systemic circulation, an increase in intracellular Ca²⁺ concentration ([Ca²⁺]i) in pulmonary arterial smooth muscle cells (PASMCs) promotes contraction through the activation of MLCK (21). Alternatively, inhibition of MLCP elicits contraction by increasing MLC phosphorylation (10). This increase in MLC phosphorylation and contraction occurring independent of changes in VSM [Ca²⁺]i is referred to as Ca²⁺ sensitization. In addition to Ca²⁺ sensitization, dynamic reorganization of the actin cytoskeleton is crucial for force generation in smooth muscle (18, 49) at actin-myosin cross bridges and force transmission to the plasma membrane, where cortical actin filaments bind to and reinforce integrins and focal adhesion proteins (21). We and others have previously shown that the RhoA/Rho kinase (ROCK) pathway is an important mediator of vasoconstriction in pulmonary hypertension (3, 4, 24, 38), and ROCK inhibitors significantly reduce pulmonary arterial pressure in CH rats (13, 19, 33, 35). ROCK directly phosphorylates and inhibits the regulatory myosin phosphatase-targeting subunit of MLCP (MYPT1), thereby increasing MLC phosphorylation. However, ROCK also increases levels of polym-
eralized actin via phosphorylation of LIM kinase (LIMK) and subsequently cofilin. Cofilin plays an essential role in regulating actin dynamics, and its activities are regulated by the phosphorylation state of its NH2-terminal serine 3 (18). In the dephosphorylated (active) state, cofilin binds actin filaments and severs them. However, serine 3 phosphorylation blocks the ability of cofilin to bind filamentous actin (F-actin), thereby preventing its actin filament disassembly activity (46). Consequently, phosphorylated (inactive) cofilin is usually associated with increased actin phosphorylation, although active cofilin has also been shown to contribute to actin polymerization by increasing the available pool of actin monomers (2, 50). Furthermore, RhoA is known to activate the formin molecule mDia, which directly catalyzes actin nucleation and polymerization (47). Therefore, the primary aim of this study was to test the hypothesis that actin polymerization contributes to augmented basal and agonist-induced pulmonary arterial constriction after CH, occurring via the RhoA/ROCK signaling pathway.

Endogenous reactive oxygen species (ROS) are important regulators of pulmonary VSM phenotype and contractility after CH. Although overall ROS generation has been reported to be reduced in isolated lungs from CH rats (39), chronic ROS inhibition with the superoxide dismutase mimetic temtop inhibits right ventricular hypertrophy and pulmonary arterial remodeling in CH rats (12) and both ROS production and vascular remodeling in CH mice (32). ROS have also been implicated in the vasoconstrictor component of CH-induced pulmonary hypertension (17, 20, 29), with superoxide anion (O2-) mediating enhanced RhoA activity and ROCK-mediated vasoconstrictor responsiveness to ET-1 in small pulmonary arteries from CH rats (24). However, whether ROS contribute to CH-induced basal pulmonary arterial tone after CH is unknown and represents an additional focus of the present study.

**METHODS**

All protocols were approved by the Institutional Animal Care and Use Committee of the University of New Mexico Health Sciences Center and abide by National Institutes of Health guidelines for animal use.

**Experimental Groups**

Adult male Sprague-Dawley rats (~12 wk old, Harlan Industries) were randomly divided between control and hypoxic groups. Animals within the hypoxic group were maintained for 4 wk in a hypobaric chamber at 0.5 atm barometric pressure (Pb) (Pb ~380 mmHg and ambient PO2 ~80 mmHg) (41). The chamber was opened 3 times/wk to provide animals with food, water, and clean bedding. Control animals were maintained at ambient Pb (Pb ~630 mmHg and ambient PO2 ~132 mmHg, Albuquerque, NM) for a similar duration. All animals had ab libitum access to food and water and were housed on a 12:12-h light-dark cycle.

**Isolated Small Pulmonary Artery Preparation**

Small pulmonary arteries were isolated and cannulated for the simultaneous assessment of vasoreactivity and vessel wall [Ca2+], as previously described (3). Rats were anesthetized with pentobarbital sodium (200 mg/kg ip), and the left lung was removed and immediately placed in ice-cold physiological saline solution (PSS) containing the following (in mM): 129.80 NaCl, 5.40 KCl, 0.83 MgSO4, 19.00 NaHCO3, 1.80 CaCl2 and 5.50 glucose. A pulmonary artery [100–200-μm internal diameter (ID), fourth-fifth order] of ~1-mm length was dissected free and transferred to a vessel chamber (CH-1, Living Systems) containing ice-cold PSS. The proximal end of the artery was cannulated with a tapered glass pipette, secured in place with a single strand of silk ligature, and gently flushed with PSS to remove any blood from the lumen. The vessel lumen was rubbed with a strand of moose mane to disrupt the endothelium before securing the distal end of the artery onto a second cannula. These microcannulae were created from borosilicate glass tubes [Sutter Instruments, outer diameter (OD) 1.2 mm and ID 0.69 mm] that were tapered to an approximate OD of 100 μm (Sutter Instruments P-30 Vertical Micropipette Puller). The vessel was stretched longitudinally to approximate in situ length and initially pressurized with PSS in the lumen to 12 mmHg with a servo-controlled peristaltic pump (Living Systems). All arteries were studied under no-flow conditions, achieved by closing the distal stopcock. Arteries were required to hold a steady pressure on switching off the servo-control function to verify the absence of leaks; any vessel with apparent leaks was discarded. The vessel chamber was transferred to the stage of an inverted microscope (Nikon Eclipse TS100), and the vessel was continuously superfused with PSS equilibrated with a gas mixture containing 10% O2, 6% CO2, and balance N2 (37°C). A vessel chamber cover was positioned to permit this same gas mixture to flow over the top of the chamber bath. We have previously reported that this gas mixture yields an approximate superfusate pH of 7.40, PO2 of 57 mmHg, and PCO2 of 31 mmHg (22). Bright-field videos of vessels were obtained with an IonOptix camera (CCD100M). Dimensional analysis was performed by IonOptix IonWizard software to measure vessel ID. To determine vessel viability, endothelium-disrupted arteries were preconstricted with UTP (5 μM, Sigma) to ~30% of baseline ID. A lack of a vasodilatory response to acetylcholine (10 μM, Sigma) confirmed endothelial disruption.

**Measurement of Vessel Wall [Ca2+]i**

Pressurized vessels were loaded abuminally with the cell-permeable Ca2+-sensitive fluorescent indicator fura-2 AM (Invitrogen) for 45 min at room temperature in the dark followed by a 20-min washout period. Fura-2-loaded vessels were alternately excited at 340 and 380 nm at a frequency of 1 Hz with an IonOptix Hyperswitch dual excitation light source (IonOptix), and the respective 510-nm emissions were collected with a photomultiplier tube. Background-subtracted 340-to-380-nm emission ratios were calculated with IonOptix IonWizard software and collected continuously throughout the experiment. Vessel wall [Ca2+], was expressed as the ratio of background-subtracted 510-nm emission fluorescence intensity attributable to excitation at 340 nm over excitation at 380 nm (F340/F380).

**Isolated Vessel Protocols**

*Role of ROS and actin polymerization in the development of basal tone after CH.* We have previously documented a critical contribution of O2 to enhanced ET-1-mediated vasoconstrictor reactivity after CH (24). However, whether O2 similarly contributes to increased basal pulmonary arterial tone in this setting is unknown. To address this possibility, after initially pressurizing the artery to 12 mmHg as described above, we determined pressure-dependent vasoconstriction by exposing isolated arteries from control and CH rats to a series of 10-mmHg pressure steps, beginning at 5 mmHg and reaching a maximum of 45 mmHg in Ca2+-replete PSS. Each pressure step lasted 5 min. Passive diameter was determined by repeating the pressure steps after 1 h of superfusion with Ca2+-free PSS [containing (in mM) 129.80 NaCl, 5.40 KCl, 0.83 MgSO4, 19.00 NaHCO3, 1.80 CaCl2 and 5.50 glucose, and 3.00 EGTA] and after flushing the lumen with Ca2+-free PSS followed by the reestablishment of no-flow conditions. Basal arterial constriction was calculated as the difference in ID between Ca2+-free and Ca2+-replete conditions expressed as a percentage of ID in Ca2+-free PSS at each pressure. Experiments were conducted in the presence of the O2 scavenger tiron (10 mM, Sigma) (3, 24) or vehicle (PSS) to determine the role of ROS in this response.
Vasoconstrictor responses to increasing concentrations of ET-1 of the actin profile of control and CH vessels, we performed additional fluorescence microscopy for F-actin and G-actin in intact pulmonary arteries from control and CH rats.

Pulmonary Arteries

Fluorescence Microscopy for F-actin and G-actin in Intact Pulmonary Arteries

Because isolated vessel experiments do not provide a direct measure of the actin profile of control and CH vessels, we performed additional experiments to determine whether exposure to CH enhances levels of polymerized actin in pulmonary arteries. Relative actin polymerization levels can be expressed F-actin-to-globular actin (G-actin) ratios, so a fluorescence microscopy approach was used to image and quantify relative F-actin and G-actin levels within pulmonary arteries. Pulmonary arteries (fourth-fifth order) were isolated, cannulated, and subjected to ET-1-dependent vasoconstriction under Ca2+-permeabilized conditions as described above. After the conclusion of the vessel study, the vessel was depressurized by removal from the vessel chamber in the continued presence of ET-1, snap frozen in liquid nitrogen, and stored at −80°C until ready to use for F-actin and G-actin labeling.

On the day of labeling, arteries were fixed in ice-cold 4% paraformaldehyde, rinsed in ice-cold PBS, and permeabilized (0.2% Triton X-100, 30 min at 4°C). Arteries were incubated overnight at 4°C in the presence of Alexa Fluor phallolidin-568 (5 U/ml, ThermoFisher) to stain F-actin filaments and Alexa Fluor DNAse-488 (9 μg/ml, ThermoFisher) to label G-actin followed by incubation with the nuclear stain TO-PRO3 (0.5 μM, 20 min, room temperature, ThermoFisher). Vessels were then washed in ice-cold PBS, mounted on poly-L-lysine slides (ThermoFisher) with Prolong Gold Antifade Mountant (ThermoFisher), and coverslipped. Slides were stored at 4°C in the dark until image analysis was performed.

Arteries were imaged using a Leica laser-scanning microscope (TCS SP5) equipped with a ×63 glycerol immersion objective (n= 5–7/group. *P < 0.05 vs. control.}

**Fluorescence Microscopy for F-actin and G-actin in Intact Pulmonary Arteries**

**Contribution of actin polymerization to ET-1-induced vasoconstriction after CH.** To evaluate mechanisms of ET-1-dependent vasoconstriction independent of changes in vessel wall [Ca2+]i, we clamped [Ca2+]i, in some arteries by permeabilizing with the Ca2+ ionophore ionomycin (3 μM, Sigma), as previously described (36). All Ca2+-permeabilized vessels were equilibrated at an internal pressure of 12 mmHg with PSS containing a calculated free Ca2+ concentration of 300 nM as previously described (24). This concentration of Ca2+ was chosen to provide optimal vasoreactivity to ET-1 while having minimal effects on basal Ca2+-dependent vasoconstriction based on a previous study (24). Vasoconstrictor responses to increasing concentrations of ET-1 (10−10–10−7 M) were measured in Ca2+-clamped vessels in the presence and absence of CytoB or vehicle. Fura-2 ratios were monitored throughout all experiments to confirm Ca2+ clamp.

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F-actin and G-actin levels in pulmonary arteries from each group. First, pulmonary artery branches were isolated from control and CH rats in HBSS (GIBCO) supplemented with 20 μM CaCl$_2$ and 1% penicillin-streptomycin. Unpressurized vessels were transferred to clean 1.5-ml tubes containing HEPES buffer + vehicle, fasudil (10 μM, Calbiochem), tiron (10 mM, Sigma), small-molecule inhibitor of formin homology 2 domain (SMIFH2; 20 μM, Sigma), or CytB (1 μM, Enzo). Vessels were incubated at 37°C for 15 min before the addition of vehicle or ET-1 (10 nM, Sigma) for another 10 min at 37°C. Vessels then were snap frozen in liquid nitrogen and stored at −80°C before homogenization. Actin cellular fractionation was performed per the protocol in the G-Actin/F-Actin In Vivo Assay Biochem Kit (no. BK037, Cytoskeleton). Briefly, vessels were homogenized with glass homogenizers in 200 μl of F-actin stabilization buffer supplemented with protease inhibitors (PIC02, Cytoskeleton) and phosphatase inhibitor cocktail (P0044, Sigma). After lysates had been cleared, 50 μl of the homogenate were saved for collagen Western blots. The remaining lysate was centrifuged at 100,000 × g at 4°C for 1 h. Blots were probed with a pan-actin monoclonal antibody. 

**Actin Cellular Fractionation and Western Blots**

To complement fluorescence microscopic analysis of F-actin-to-G-actin ratios, a cellular fractionation protocol was used to measure F-actin and G-actin levels in pulmonary arteries from each group. First, pulmonary artery branches were isolated from control and CH rats in HBSS (GIBCO) supplemented with 20 μM CaCl$_2$ and 1% penicillin-streptomycin. Unpressurized vessels were transferred to clean 1.5-ml tubes containing HEPES buffer + vehicle, fasudil (10 μM, Calbiochem), tiron (10 mM, Sigma), small-molecule inhibitor of formin homology 2 domain (SMIFH2; 20 μM, Sigma), or CytB (1 μM, Enzo). Vessels were incubated at 37°C for 15 min before the addition of vehicle or ET-1 (10 nM, Sigma) for another 10 min at 37°C. Vessels then were snap frozen in liquid nitrogen and stored at −80°C before homogenization. Actin cellular fractionation was performed per the protocol in the G-Actin/F-Actin In Vivo Assay Biochem Kit (no. BK037, Cytoskeleton). Briefly, vessels were homogenized with glass homogenizers in 200 μl of F-actin stabilization buffer supplemented with protease inhibitors (PIC02, Cytoskeleton) and phosphatase inhibitor cocktail (P0044, Sigma). After lysates had been cleared, 50 μl of the homogenate were saved for collagen Western blots. The remaining lysate was centrifuged at 100,000 × g at 4°C for 1 h. Blots were probed with a pan-actin monoclonal antibody.
primary antibody (AAN01, Cytoskeleton) in Tris-buffered saline with 0.1% Tween 20 with 0.1% milk at 4°C overnight and horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:3,000, no. 172-1019, lot no. 64026773, Bio-Rad) for 1 h at room temperature the next day. Bands were developed with ECL Western blotting substrate (no. 32209, Thermo Scientific), and densitometry quantification was performed using ImageJ (National Institutes of Health).

**Phosphorylated Cofilin/Cofilin Western Blots**

Because ROCK is known to stimulate actin polymerization indirectly through LIMK phosphorylation and subsequent cofilin phosphorylation, an immunoblot method was used to measure both phosphorylated (p)-cofilin and total cofilin levels in pulmonary artery lysates. Lysates were collected as described above. Total protein extracts were separated by SDS-PAGE and blotted using anti-cofilin phospho-serine 3 (1:500, no. 3311S, lot no. 6, Cell Signaling) and anti–β-actin (1:14,000, no. ab8227, lot no. GR186254-1, Abcam). p-Cofilin blots were stripped and reprobed with anti-cofilin primary antibody (1:5,000, no. ab42824, lot no. GR295201-1, Abcam) to assess total cofilin levels. All bands were detected with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody and ECL as previously described. Densitometry was performed using ImageJ. Values are expressed as ratios of p-cofilin to total cofilin.

**Statistical Analysis**

All data are expressed as means ± SE. A t-test or two-way ANOVA was used to make comparisons as appropriate. Data expressed as proportions were arcsine transformed before statistical analysis. A Student-Newman-Keuls post hoc test was used when differences were detected by ANOVA. *P < 0.05 was accepted as statistically significant.

**RESULTS**

**ROS Contribute to CH-Induced Basal Vasoconstriction**

In agreement with a previous study (4), CH vessels exhibited significantly elevated basal constriction compared with control vessels at intraluminal pressures of 15–45 mmHg (Fig. 1A). This vasoconstrictor response in CH arteries was not associated

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**Fig. 5.** Endothelin-1 (ET-1) stimulates actin polymerization in pulmonary arteries from chronic hypoxia (CH) rats. A: representative fluorescence images of DNaseI 488 (green; G-actin), phalloidin 555 (red; F-actin), and TO-PRO (blue; nuclei) in pulmonary arteries from control and CH rats treated with ET-1 (10 nM) or vehicle. Drawings on the right indicate artery orientation. B: fluorescence intensity was quantified to determine F-actin-to-G-actin ratios. n = 4/group. *P < 0.05 vs. CH vehicle.
with an increase in vessel wall [Ca\(^{2+}\)], (Fig. 1B). Neither baseline ID (control: 165 ± 14 μm and CH: 147 ± 14 μm, 12-mmHg intraluminal pressure) nor fura-2 emission ratios (Fig. 1B) differed significantly between groups.

Pressure-dependent constriction in CH arteries was abolished by the O\(_2\) scavenger tiron (3, 36), supporting a contribution of ROS to this response (Fig. 2A). On the basis of a previous study indicating that control arteries do not develop basal tone (4), experiments with tiron were conducted solely in arteries from CH rats. Tiron was without effect on [Ca\(^{2+}\)], as assessed by fura-2 ratios (data not shown).

**Actin Polymerization Contributes to Basal Pressure-Dependent Vasoconstriction and ET-1-Induced Vasoconstrictor Reactivity After CH**

Similar to effects of tiron, the actin polymerization inhibitors LatB and CytB blocked the development of arterial constriction in response to increasing intraluminal pressure (Fig. 2B). Fura-2 ratios were unaltered by administration of actin polymerization inhibitors (data not shown).

To directly evaluate the role of actin polymerization in ET-1-induced vasoconstriction independent of changes in [Ca\(^{2+}\)], we performed cumulative concentration-response curves to ET-1 in arteries from each group that had been permeabilized to Ca\(^{2+}\) using ionomycin to clamp [Ca\(^{2+}\)], (23, 24). Resting ID did not differ between control (156 ± 18 μm) or CH arteries (153 ± 12 μm). In agreement with our previous study (24), vasoconstrictor sensitivity to ET-1 (10\(^{-8}\)–10\(^{-7}\) M) was greater in Ca\(^{2+}\)-permeabilized arteries from CH rats compared with control vessels (Fig. 3A). Fura-2 ratios did not increase in response to ET-1 and were similar between groups, confirming the efficacy of ionomycin to clamp [Ca\(^{2+}\)], (Fig. 3B). Consistent with effects of actin polymerization inhibitors to prevent CH-induced basal arterial constriction (Fig. 2B), CytB significantly blunted the vasoconstrictor response to ET-1 in CH vessels (Fig. 4B). In contrast, CytB was without effect on responses to ET-1 control pulmonary arteries (Fig. 4A). Collectively, these data suggest that actin polymerization contributes to both pressure-dependent and ET-1-induced vasoconstriction selectively in arteries from CH rats.

**ROS, ROCK, and mDia Mediate ET-1-Induced Actin Polymerization After CH**

Effects of ET-1 to stimulate actin polymerization were compared between small pulmonary arteries from control and CH rats using fluorescent molecular labeling techniques to quantify the ratio of F-actin to G-actin levels (Fig. 5A). Although F-actin-to-G-actin ratios were not different between arteries from control and CH rats under basal conditions, ET-1 produced a significant increase in the F-actin-to-G-actin ratio after CH (Fig. 5B) but not in control arteries.

In agreement with these findings from fluorescent staining protocols, actin fractionation assays demonstrated no differences in basal F-actin-to-G-actin ratios between control and CH vessels, but treatment with ET-1 significantly enhanced actin polymerization in CH pulmonary arteries compared with both CH vehicle-treated arteries and control ET-1-treated arteries (Fig. 6). Pretreatment with the ROCK-specific inhibitor fasudil, the ROS scavenger tiron, the mDia inhibitor SMIFH2, or CytB prevented this response to ET-1 in CH arteries while having no effect on control vessels (Fig. 6).

Cofilin is an actin disassembly factor that is inhibited by LIMK-dependent phosphorylation, contributing to agonist-induced actin polymerization and smooth muscle contraction (1, 18, 46). To determine whether ET-1-induced actin polymerization in CH arteries correlates with cofilin phosphorylation, we measured levels of p-cofilin and total cofilin protein by Western blot analysis in pulmonary artery lysates from each group. No differences in p-cofilin-to-cofilin ratios were detected between control and CH vessels (Fig. 7, A and B) in any treatment group, but overall levels of both p-cofilin and cofilin were higher in CH vessels (Fig. 7, C and D). Neither the ratio of p-cofilin to cofilin nor levels of p-cofilin or total cofilin were altered by ET-1, fasudil, tiron, SMIFH2, or CytB. Taken together, these data indicate that ROS, ROCK, and mDia mediate ET-1-induced actin polymerization after CH, but this is not dependent on greater p-cofilin-to-cofilin ratios.

**DISCUSSION**

The overall goal of this study was to test the hypothesis that actin polymerization contributes to augmented basal and...
nirst-induced pulmonary arterial vasoconstriction after CH downstream of the ROS/RhoA/ROCK signaling pathway. Our results indicate that 1) actin polymerization was required for enhanced vasoconstrictor responsiveness in isolated vessels from rats exposed to CH, both at baseline and in response to ET-1; 2) stimulation with ET-1 increased actin polymerization as measured by the F-actin-to-G-actin ratio in pulmonary arteries from CH, but not control, animals; and 3) scavenging of \( \mathrm{O}_2^- \) and pharmacological inhibition of ROCK and mDia prevented ET-1-induced actin polymerization in pulmonary arteries from CH animals, independent of alterations in p-cofilin-to-cofilin ratios. Collectively, these findings suggest that actin polymerization contributes to augmented pulmonary vasoreactivity after CH in a ROS- and ROCK-dependent manner (see Fig. 8).

Previous reports have indicated that \( \mathrm{O}_2^- \) can activate RhoA in PASMCs (28) and that CH facilitates the coupling of receptor stimulation or depolarizing stimuli to RhoA activation and subsequent \( \mathrm{Ca}^{2+} \) sensitization (3, 24). Although an earlier study from our laboratory demonstrated an effect of CH to induce pressure-dependent tone in small pulmonary arteries that is similarly RhoA mediated (4), the contribution of ROS to this response is unknown. Considering that increasing pressure in CH arteries leads to PASMC membrane depolarization independent of \( \mathrm{Ca}^{2+} \) influx, we presently set out to address the potential contribution of ROS to basal tone in arteries from pulmonary hypertensive rats. In this study, the \( \mathrm{O}_2^- \) scavenger tiron was used to deplete ROS generated in arterioles isolated from control and CH rats. Consistent with previous studies implicating ROS in augmented agonist- and depolarization-induced pulmonary arterial constriction after CH (3, 24), tiron significantly blunted the vasoconstriction observed in CH arteries, confirming that \( \mathrm{O}_2^- \) generation is necessary for hypoxia-induced basal vasoconstriction.

ROS have previously been shown to mediate \( \mathrm{Ca}^{2+} \) sensitization in pulmonary VSM after CH in a RhoA/ROCK-dependent manner (3, 24, 28). Elevated ROS after CH augment ET-1-induced pulmonary artery vasoconstriction, and this response is attenuated, but not abolished, by pharmacological ROCK inhibition and MLCK inhibition (24). Furthermore, ET-1-induced increases in MYPT1 phosphorylation were significantly reduced by ROCK inhibition (24). These results suggest that pulmonary vasoconstriction after CH is at least partially mediated by ROCK-dependent phosphorylation and inactivation of MYPT1 and subsequent increased activity of MLC and PASMC contraction.

It has also been well documented that the RhoA/ROCK pathway lies upstream of actin polymerization (31), and polymerization of actin filaments is important for contraction and force generation in smooth muscle (46). The role of filamentous actin in actomyosin cross-bridge cycling is well established and widely recognized as the primary mechanism for tension development and shortening in muscle cells, but a more recent study has highlighted a role for actin polymerization and cytoskeletal dynamics in smooth muscle contraction that extend beyond actomyosin interactions and cross-bridge cycling (18). The pharmacological agents CyTb and LatB, which inhibit actin polymerization by capping the ends of growing actin filaments and sequestering free globular actin monomers, respectively, have been widely used to assess the role of actin polymerization in smooth muscle cell contraction. These inhibitors significantly inhibit contractility and force generation.
in airway smooth muscle (34, 48), systemic VSM (5, 40), and uterine and intestinal smooth muscle (37, 42). Fediu et al. (15, 16) also demonstrated a role for actin polymerization-mediated constriction in pulmonary arteries in response to thromboxane in a porcine model of persistent pulmonary hypertension of the newborn. Much of this evidence suggests that actin polymerization occurring in a submembranous region of the smooth muscle cell enhances contractility by more strongly transmitting tension generated by cross-bridge cycling (14, 18, 49). On the basis of these studies, RhoA/ROCK-induced actin polymerization may provide an alternative parallel pathway to cause contraction independent of changes in \( [\text{Ca}^{2+}]_i \) and MLC activity in pulmonary arteries.

Pharmacological treatment with either CytB or LatB abolished CH-induced basal vasoconstriction, and pretreatment with CytB prevented the augmented pulmonary artery vasoconstrictor response to ET-1 that has previously been observed after CH (24). Taken together, these results suggest that actin polymerization is required for elevated basal pulmonary arterial tone and ET-1 vasoconstrictor sensitivity after CH exposure. Because actin polymerization measurements are complicated by the dynamic nature of the actin cytoskeleton, F-actin-to-G-actin ratios were used as a proxy for relative levels of polymerized actin. Interestingly, both immunofluorescent and cellular fractionation methods determined that pulmonary arteries from CH animals show elevated actin polymerization levels in response to acute ET-1 treatment but not at baseline. This lack of difference in baseline F-actin-to-G-actin ratios between control and CH vessels may be due to the fact that the pulmonary arteries were not pressurized before ET-1 stimulation, and pressure may be a stimulus for actin polymerization. Alternatively, PASMC contraction may exhibit a greater dependency on levels of polymerized actin after CH, analogous to its dependence on ROCK-dependent \( [\text{Ca}^{2+}]_i \) sensitization. ROCK does not play an appreciable role in either depolarization-stimulated or ET-1-induced constriction in control arteries. However, on the basis of previous studies, CH allows coupling of these stimuli to ROS- and ROCK-dependent constriction (3, 4, 23, 24). In the present study, the acute development of tone in response to intraluminal pressure and the significant increase in actin polymerization in response to acute exposure to ET-1 point to a very rapid, dynamic actin polymerization response in CH vessels. Importantly, this response was abolished by scavenging of \( \text{O}_2^- \) with tiron, pharmacological inhibition of ROCK and mDia with fasudil and SMIFH2, respectively, or inhibition of actin polymerization with CytB. These results suggest that the actin polymerization response to ET-1 is dependent on ROS and two RhoA effectors, ROCK and mDia.

mDia directly promotes actin polymerization by nucleating filaments in concert with profilin (47). Alternatively, ROCK activation leads to actin polymerization through an indirect method. ROCK phosphorylates LIMK, which then phosphorylates cofilin (1). Cofilin is known to sever actin filaments, but, when it becomes phosphorylated (inactivated), these activities
are inhibited, allowing actin filaments to extend. However, dephosphorylated (active) cofilin also contributes to actin filament assembly by replenishing the globular actin monomers that are necessary for polymerization (2). Zhao et al. (50) previously showed that stimulation of tracheal smooth muscle with acetylcholine causes contraction via dephosphorylation of cofilin. When a constitutively phosphorylated cofilin mutant, cofilin S3E, is overexpressed, acetylcholine is unable to stimulate actin polymerization and contraction because of the lack of available actin monomers. Traditionally, an elevated p-cofilin-to-cofilin ratio indicates signaling through the ROCK-LIMK-cofilin pathway. To test the hypothesis that CH-induced actin polymerization is dependent on this pathway, Western blots were performed to measure levels of p-cofilin and cofilin in pulmonary artery homogenates. Surprisingly, there were no differences in p-cofilin-to-cofilin ratios between control and CH pulmonary arteries, although others have previously reported a less robust relationship between cofilin phosphorylation and actin polymerization in pulmonary arteries than expected (15). In contrast, we observed overall higher levels of both p-cofilin and cofilin in CH vessels compared with control vessels, indicative of a larger pool of cofilin, which may more readily respond to stimuli. Upregulation of cofilin expression has previously been observed by Dai et al. (9) in a monocrotaline model of pulmonary hypertension, where it was proposed that high levels of cofilin correspond to a more dynamic subcortical actin network in motile PASMCs. This high level of cofilin expression may also explain why there was no basal elevation in polymerized actin levels in CH vessels and why the polymerization response to ET-1 was so rapid. Finally, because inhibition of mDia significantly attenuated actin polymerization in CH arteries, and the phosphorylation state of cofilin has no regulatory effect on mDia activity, it appears that the actin polymerization response to ET-1 may be mediated by the combined influences of these distinct signaling pathways.

In conclusion, this study supports a previously undescribed role of actin polymerization to mediate CH-induced basal pulmonary arterial tone and vasoreactivity to ET-1 in a ROS-, ROCK-, and mDia-dependent manner. These results advance our understanding of the signaling mechanisms involved in CH-induced vasoconstriction and associated pulmonary hypertension. Our results suggest that inhibition of ROCK may target both Ca\(^{2+}\) sensitization and actin polymerization pathways, either in series or in parallel. Actin polymerization may represent a component of Ca\(^{2+}\) sensitization because it may provide greater interaction between myosin and actin, such that a greater contraction is achieved for a given level of Ca\(^{2+}\). Furthermore, because mDia lies parallel to and not downstream of ROCK, inhibition of mDia-mediated actin polymerization may provide an additional target for reducing vasoconstrictor responsiveness after CH.

Future studies should address whether this signaling pathway is active in response to other vasoreactive stimuli. This novel finding that actin polymerization plays a particular role in CH-induced pulmonary vasoconstriction leads to some interesting questions left to be answered. Traditionally, actin polymerization in the vasculature has been studied in the context of smooth muscle cell contractility. The actin that interacts with myosin generates tension and cell shortening and is usually referred to as contractile actin (49). Clearly, some basal level of actin polymerization is needed to form actin-myosin cross bridges, and this is a very stable group of filaments. Previous studies have suggested that the actin already bound to tropomyosin at cross bridges is resistant to cofilin and at least partially protected against disassembly by cytochalasin and latrunculin (8, 25). It is unlikely that the CytB treatments performed in this study targeted depolymerization of these already-existing actin filaments for two reasons: 1) F-actin-to-G-actin ratios in CytB-treated pulmonary artery lysates were not different from vehicle-treated vessels (Fig. 6) and 2) CytB-treated control pulmonary arteries constricted in response to ET-1 in a manner similar to vehicle-treated vessels (Fig. 4). However, it is possible that after CH, either ET-1 treatment or increased vascular pressure induces the assembly of additional contractile units by increasing contractile actin polymerization. Such activities would be especially sensitive to the actin polymerization inhibitors CytB and LatB because they prevent assembly of new actin filaments. Alternatively, ET-1 treatment after CH exposure may instead strengthen force transmission of already-existing contractile units to the extracellular matrix by increasing actin polymerization near the cell cortex (see Fig. 8), which has been observed in other studies in VSM (14, 26, 27). It is possible that exposure to CH relies on this dynamic actin pool for elevated arterial tone and sensitivity to agonists. Additional studies examining actin isoform levels and actin filament subcellular localization will be needed to address this question.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS


REFERENCES


