Chronic hypoxia induces Rho kinase-dependent myogenic tone in small pulmonary arteries

Brad R. S. Broughton, Benjimen R. Walker, and Thomas C. Resta

Vascular Physiology Group, Department of Cell Biology and Physiology, University of New Mexico Health Sciences Center, Albuquerque, New Mexico

Submitted 1 July 2007; accepted in final form 4 February 2008

Broughton BR, Walker BR, Resta TC. Chronic hypoxia induces Rho kinase-dependent myogenic tone in small pulmonary arteries. Am J Physiol Lung Cell Mol Physiol 294: L797–L806, 2008. First published February 8, 2008; doi:10.1152/ajplung.00253.2007.—Myogenic tone in the pulmonary vasculature of normoxic adult animals is minimal or nonexistent. Whereas chronic hypoxia (CH) increases basal tone in pulmonary arteries, it is unclear if a portion of this elevated tone is due to development of myogenicity. Since basal arterial RhoA activity and Rho kinase (ROK) expression are augmented by CH, we hypothesized that CH elicits myogenic reactivity in pulmonary arteries through ROK-dependent vascular smooth muscle (VSM) Ca\(^{2+}\) sensitization. To test this hypothesis, we assessed the contribution of ROK to basal tone and pressure-induced vasoconstriction in endothelium-disrupted pulmonary arteries (50–300 μm inner diameter [ID]) from control and CH [4 wk at 0.5 atmosphere (atm)] rats. Arteries were loaded with fura-2 AM to continuously monitor VSM intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)). Basal VSM [Ca\(^{2+}\)]\(_{i}\) was not different between groups. The ROK inhibitor, HA-1077 (100 nM to 30 μM), caused a concentration-dependent reduction of basal tone in CH arteries but had no effect in control vessels. In contrast, PKC inhibition with GF109203X (1 μM) did not alter basal tone. Furthermore, significant vasoconstriction in response to stepwise increases in intraluminal pressure (5–45 mmHg) was observed at 12, 15, 25, and 35 mmHg in arteries (50–200 μm ID) from CH rats. This myogenic reactivity was abolished by HA-1077 (10 μM) but not by GF109203X. VSM [Ca\(^{2+}\)]\(_{i}\), was unaltered by HA-1077, GF109203X, or increases in pressure in either group. Myogenicity was not observed in larger vessels (200–300 μm ID). We conclude that CH induces myogenic tone in small pulmonary arteries through ROK-dependent myofilament Ca\(^{2+}\) sensitization.

The mechanisms leading to increased VSM tone in response to CH and the subsequent development of pulmonary hypertension remain unclear. Although augmented basal tone in pulmonary arteries from CH rats may be a function of greater Ca\(^{2+}\) entry though nonselective cation channels in pulmonary VSM (33, 62), an additional possibility is that CH increases the sensitivity of the contractile apparatus to Ca\(^{2+}\) to mediate this response. Indeed, myofilament Ca\(^{2+}\) sensitization via activation of the RhoA/Rho kinase (ROK) pathway and consequent inhibition of myosin light-chain phosphatase (MLCP) represents a central component of VSM contraction. In agreement with this possibility are studies by Nagaoka and colleagues (40, 41), who recently demonstrated that the ROK inhibitor, Y-27632, dramatically reduced both mean pulmonary arterial pressure and total pulmonary resistance in CH rats that had been acutely returned to normoxia. Furthermore, previous data from our laboratory indicate that CH augments RhoA/ROK induced pulmonary VSM Ca\(^{2+}\) sensitization, a response associated with enhanced pulmonary arterial RhoA and ROK activity (25). However, it remains to be determined whether this contribution of ROK to CH-induced pulmonary hypertension is mediated by an endothelium-derived paracrine factor, by ROK-dependent alterations in endothelial control of VSM tone, or rather represents a mechanism intrinsic to the VSM.

The myogenic response, defined as vasoconstriction in response to increasing transmural pressure, contributes to resting vascular tone and autoregulation of blood flow in the systemic circulation. Although the mechanism by which VSM stretch mediates contraction involves depolarization-induced Ca\(^{2+}\) influx through L-type voltage-dependent Ca\(^{2+}\) channels (21), a contribution of ROK-mediated VSM Ca\(^{2+}\) sensitization to myogenic behavior has additionally been demonstrated in systemic vascular preparations (6, 13, 20, 30, 59, 60). However, whether myogenic behavior explains the elevated basal pulmonary arterial tone following CH has not been addressed and represents a focus of the present investigation.

The current study examined the hypothesis that CH induces pulmonary myogenic tone via ROK-dependent VSM Ca\(^{2+}\) sensitization. To test this hypothesis, we assessed the contribution of ROK to basal tone in endothelium-disrupted, pressurized small pulmonary arteries from control and CH rats. Our findings demonstrate a novel role for CH to induce pulmonary arterial myogenic tone through a ROK-dependent myofilament Ca\(^{2+}\) sensitization signaling mechanism.

Address for reprint requests and other correspondence: T. C. Resta, Dept. of Cell Biology and Physiology, Univ. of New Mexico Health Sciences Center, MSC08 4750, 1 Univ. of New Mexico, Albuquerque, NM 87131-0001 (e-mail: tresta@salud.unm.edu).

http://www.ajplung.org 1040-0605/08 $8.00 Copyright © 2008 the American Physiological Society
METHODS

All protocols and surgical procedures employed in this study were reviewed and approved by the Institutional Animal Care and Use Committee of the University of New Mexico Health Sciences Center (Albuquerque, NM).

Experimental Groups

Male Sprague-Dawley rats were exposed to CH by placement in a hypobaric chamber maintained at 380 mmHg for 4 wk as previously described (15, 16, 24–26, 42, 45–48). Control rats were maintained in the same facility under normobaric (630 mmHg) conditions.

Cannulation of Small Pulmonary Arteries for Dimensional Analysis

Rats were anesthetized with sodium pentobarbital (200 mg/kg ip), and the left lung was removed and immediately placed in ice-cold physiological saline solution (PSS) containing (in mM) 129.8 NaCl, 5.4 KCl, 0.83 MgSO4, 19 NaHCO3, 1.8 CaCl2, and 5.5 glucose. A pulmonary artery [50–300 μm inner diameter (ID)] of ~1 mm length and without side branches was dissected free and transferred to a vessel chamber (CH-1, Living Systems) containing ice-cold PSS. Arteries were then cannulated and pressurized to 12 mmHg as previously described (24–26). Any vessels with apparent leaks were discarded. The vessel chamber was transferred to the stage of a Nikon Eclipse TS100 microscope, and the preparation was superperfused with aerated PSS (37°C). Bright-field images of vessels were obtained with an IonOptix CCD100M camera, and dimensional analysis was performed by IonOptix SarcLen software to measure ID as described in previous studies from our laboratory (14–16, 24–26, 42).

Measurement of VSM [Ca2+].

Pressurized arteries were loaded abuminally with the cell-permeant, ratiometric, Ca2+-sensitive fluorescent indicator fura-2 AM (Molecular Probes) for 45 min at room temperature in the dark as detailed previously (14–16, 24–26, 42). Vessels were rinsed for 20 min with aerated PSS (37°C) following the loading period to wash out excess dye and to allow for hydrolysis of AM groups by intracellular esterases. Fura-2-loaded arteries were alternately excited at 340 and 380 nm at a frequency of 10 Hz with an IonOptix Hyperswitch dual excitation light source, and the respective 510-nm emissions were collected with a photomultiplier tube. Background-subtracted 340/380 emission ratios were calculated with IonOptix IonWizard software and recorded continuously throughout the experiment with simultaneous measurement of ID from red wavelength bright-field images as described above. VSM intracellular Ca2+ concentration ([Ca2+]i) is expressed as the emission fluorescence intensity due to excitation at 340 nm (F340) to F380 mean ratio from the background-subtracted 510-nm signal.

Verification of Endothelial Integrity/Disruption

For experiments in endothelium-intact vessels, endothelial integrity was assessed before experimentation by preconstricting arteries with UTP (~30% of baseline ID) and measuring the subsequent vasodilatory response to ACh (1 μM). ACh mediates dose-dependent vasodilation and decreases in VSM [Ca2+], in this preparation as determined in preliminary experiments from our laboratory. In some experiments, the endothelium was disrupted to directly examine mechanisms of CH-induced upregulation of VSM RhoA/ROK signaling independent of endothelial influences. This was achieved by rubbing the lumen with a strand of moose mane following cannulation of the proximal end of the artery. The effectiveness of endothelial disruption was verified by the lack of a vasodilatory response to ACh (1 μM) in UTP-constricted vessels. Following administration of ACh, vessels were rinsed in normal PSS and loaded with fura-2 AM as above.

Isolated Vessel Experiments

Effect of nitric oxide on basal pulmonary arterial tone and VSM [Ca2+]i. To identify whether CH induces basal pulmonary arterial tone, we examined vasodilatory responses to the nitric oxide (NO) donor, spermine NONOate (1 μM; Cayman Chemicals), under resting conditions in endothelium-disrupted arteries. VSM [Ca2+]i levels were simultaneously measured. We have recently shown that this concentration of spermine NONOate mediates dilation in agonist- preconstricted small pulmonary arteries from both control and CH rats (25).

Effects of ROK and PKC inhibition on basal pulmonary arterial tone and VSM [Ca2+]i. To establish the contribution of ROK to elevated basal tone following CH, concentration-response curves to the selective ROK inhibitor, HA-1077 (1 nM to 30 μM; Sigma; Ref. 9), or vehicle (PSS) were performed under basal conditions in endothelium-disrupted control and CH arteries. Since VSM Ca2+ sensitization can additionally be mediated by PKC stimulation, similar protocols were performed in separate sets of vessels treated with the broad spectrum PKC inhibitor, GF109203X (1 μM; Cayman Chemicals; Ref. 58), or PSS vehicle. To verify the specificity of these inhibitors, additional experiments examined concentration-response curves to the PKC activator, PMA (10−10−10−6 M; Sigma), following pretreatment with either HA-1077 (10 μM) or GF109203X (1 μM) in arteries from each group of rats. Previous studies from our laboratory and others have demonstrated that these concentrations of HA-1077 (9) and GF109203X (25) selectively inhibit ROK and PKC.

Effect of CH on myogenic reactivity in small pulmonary arteries. To establish the myogenic character of elevated basal tone in hyperoxic pulmonary arteries, pressure-induced vasoconstrictor responses were determined by exposing both endothelium-intact and endothelium-disrupted vessels to a series of 10 mmHg pressure steps beginning at 5 mmHg and ending at 45 mmHg. Each pressure step was held for 5 min. To determine the passive diameter at each pressure step, vessels were superfused for 1 h with Ca2+-free PSS that contained (in mM) 129.8 NaCl, 5.4 KCl, 0.83 MgSO4, 19 NaHCO3, 5.5 glucose, and 3 EGTA. Another pressure-response curve was then performed under Ca2+-free conditions. Pressure-induced tone then was calculated as described below. Time control experiments were performed in separate vessels from each group by comparing successive pressure-response curves in the continued presence of Ca2+-containing PSS.

Contribution of ROK, PKC, L-type, and T-type Ca2+ channels to CH-induced myogenic tone. To examine the role of ROK, PKC, L-type, and T-type Ca2+ channels in modulating pressure-induced tone, endothelium-disrupted arteries were incubated with HA-1077 (10 μM), GF109203X (1 μM), the L-type Ca2+ channel inhibitor diltiazem (50 μM), the relatively selective T-type Ca2+ channel inhibitor mibebradil (10 μM; Ref. 32), or vehicle. Both L-type and T-type Ca2+ channels are present in pulmonary VSM (36, 49, 51) and are thought to play an important role in the myogenic response in systemic arteries (12, 21, 61). Previously, our laboratory has demonstrated that this concentration of diltiazem inhibits KCl-mediated increases in VSM [Ca2+]i, in this preparation (24, 26). In addition, several studies have shown that 10 μM mibebradil effectively inhibits T-type Ca2+ channels (49, 61). Pressure-response curves were then performed as above.

Calculations and Statistics

Vasodilator (spermine NONOate, HA-1077, and GF109203X experiments) and vasoconstrictor (PMA) responses were calculated as a percent of baseline ID. Pressure-induced tone was calculated as the % difference in ID between Ca2++-free and Ca2+-containing conditions at each pressure as we have previously described (15, 16). All data are expressed as means ± SE, and values of n refer to the number of animals in each group. A t-test, two-way ANOVA, or two-way repeated measures ANOVA was used to make comparisons when
appropriate. If differences were detected by ANOVA, individual
groups were compared with the Student-Newman-Keuls test. A prob-
ability of \( P \leq 0.05 \) was accepted as significant for all comparisons.

RESULTS

Figure 1 depicts a trace of vessel ID and VSM [Ca\(^{2+}\)] \(_{i}\) in
response to increasing concentrations to KCl in an endothelium-
disrupted control pulmonary artery. We observed a concentra-
tion-dependent increase in [Ca\(^{2+}\)] \(_{i}\) and associated vasocon-
striction. This demonstrates that our experimental preparation
is able to detect simultaneous changes in vessel ID and VSM
[Ca\(^{2+}\)] \(_{i}\) as previously reported (14–16, 24–26, 42).

Spermine NONOate Reduces Basal Tone in CH Pulmonary
Arteries Without Altering VSM [Ca\(^{2+}\)] \(_{i}\)

Spermine NONOate (1 \( \mu \)M) had little vasodilatory influence
in endothelium-disrupted control pulmonary arteries (Fig. 2A)
at a dose previously shown to cause dilation in agonist-
preconstricted control vessels (24–26). In contrast, spermine
NONOate induced potent vasodilation in CH pulmonary arter-
ies without significantly changing basal VSM [Ca\(^{2+}\)] \(_{i}\) (Fig. 2,
A and B). Furthermore, resting [Ca\(^{2+}\)] \(_{i}\) was not significantly
different between arteries from control (\( F_{340}/F_{380} = 0.93 \pm 
0.03; n = 5 \)) and CH rats (\( F_{340}/F_{380} = 1.09 \pm 0.09; n = 5 \)).
These data suggest that CH increases basal pulmonary arterial
tone via a Ca\(^{2+}\) sensitization signaling mechanism.

ROK Inhibition Decreases Both Basal Tone and Spermine
NONOate-Induced Dilation in Pulmonary Arteries from
CH Rats

The selective ROK inhibitor HA-1077 induced a concentra-
tion-dependent vasodilatory response in endothelium-disrupted small
pulmonary arteries pressurized to 12 mmHg from CH but not
control rats (Fig. 3A). Furthermore, there was no significant
change in VSM [Ca\(^{2+}\)] \(_{i}\) in response to HA-1077 in arteries from
either group (Fig. 3B), thus supporting a contribution of ROK-
dependent VSM Ca\(^{2+}\) sensitization to elevated basal tone follow-
ing CH. Spermine NONOate (1 \( \mu \)M) was additionally without
effect on either basal tone (1.84\% ± 1.00\%; \( n = 5 \)) or VSM
[Ca\(^{2+}\)] \(_{i}\) (\( \Delta F_{340}/F_{380} = 0.014 \pm 0.011; n = 5 \)) in CH arteries when
administered at the conclusion of the HA-1077 concentra-
tion-response curve, suggesting that the NO-mediated reduction in
basal tone in CH arteries observed in Fig. 2 is mediated by
inhibition of ROK-induced myofilament Ca\(^{2+}\) sensitization.

HA-1077 (10 \( \mu \)M) had no effect on vasoconstriction to the
PKC agonist, PMA, in control arteries (Fig. 4), thus demon-
strating the selectivity of HA-1077 for ROK in this preparation.
In contrast, the broad-spectrum PKC inhibitor, GF109203X
(1 \( \mu \)M), largely attenuated PMA-induced constriction in these
arteries as anticipated.

PKC Inhibition Does not Alter Basal Pulmonary Arterial
Tone Following CH

In contrast to vasodilatory influences of ROK inhibition in
CH arteries (Fig. 3A), GF109203X (1 \( \mu \)M) produced no sig-
significant vasodilation in either control (0.32% ± 0.17%, n = 5) or CH vessels (0.62% ± 0.33%, n = 5). VSM [Ca^{2+}i], was similarly unaltered by GF109203X in either group (F340/F380 = 1.02 ± 0.06 for control and 0.99 ± 0.09 for CH; n = 5/group). These data indicate that elevated basal tone in CH arteries is not a function of PKC-induced VSM Ca^{2+}i sensitization.

**CH Elicits Myogenic Tone in Small Pulmonary Arteries Independent of Changes in VSM Ca^{2+}i.**

To identify whether increased basal tone in CH arteries reflects a myogenic mechanism, pressure-induced vasoconstrictor and VSM [Ca^{2+}i] responses were assessed in endothelium-disrupted arteries from both groups over three ranges of vessel diameters (50–100, 150–200, and 200–300 μm ID). No evidence for myogenic tone was observed in arteries between 200–300 μm ID in either control or CH groups (Fig. 5A). In contrast, significant myogenic vasoconstriction was demonstrated at 12, 15, 25, and 35 mmHg in CH arteries ranging from 150 to 200 μm ID, although no such vasoactivity occurred in control arteries of the same size (Fig. 5C). More pronounced myogenicity was observed at 12, 15, 25, and 35 mmHg in CH pulmonary arteries between 50–150 μm ID. Surprisingly, significant pressure-induced tone was also present in control arteries at 15, 25, and 35 mmHg, although this response was less than in CH vessels of similar size (Fig. 5E). VSM [Ca^{2+}i], did not significantly change in response to increasing intraluminal pressure in vessels from either group, nor were differences in [Ca^{2+}i], observed between groups (Fig. 5, B, D, and F). Time control experiments in arteries within the 150–200 μm ID range revealed no differences between successive pressure-response curves performed under Ca^{2+}-replete conditions in arteries from either control (n = 5) or CH rats (n = 5; data not shown).

Significant myogenic tone was also present in endothelium-intact CH arteries at 12, 15, 25, and 35 mmHg but not in control vessels (CH vessels: 5 mmHg = −0.53% ± 1.55%, 12 mmHg = 5.76% ± 0.75%, 15 mmHg = 4.87% ± 0.96%, 25 mmHg = 5.91% ± 1.75%, 35 mmHg = 3.79% ± 1.49%, and 45 mmHg = 1.05% ± 1.14%; control vessels: 5 mmHg = −0.93% ± 0.70%, 12 mmHg = 2.04% ± 0.46%, 15 mmHg = 1.02% ± 0.92%, 25 mmHg = 0.98% ± 0.94%, 35 mmHg = −0.11% ± 0.99%, and 45 mmHg = 0.66% ± 0.94%; n = 4/group).

**ROK, but not PKC, Inhibition Abolishes Myogenic Tone in Pulmonary Arteries from CH Rats**

Since HA-1077 reduced basal tone in CH arteries, we examined the contribution of ROK to myogenic tone in 150–200 μm ID arteries from both control and CH rats. Pretreatment with HA-1077 (10 μM) was without effect in control arteries (Fig. 6A) but abolished pressure-induced tone at 12, 15, 25, and 35 mmHg in arteries from CH rats (Fig. 6C). VSM [Ca^{2+}i], was not altered by HA-1077 in vessels from either group (Fig. 6, B and D). In contrast, GF109203X (1 μM) did not significantly alter myogenic tone or VSM [Ca^{2+}i], in 150–200 μm ID arteries from either control and CH rats (Fig. 7, A–D).

**L-Type and T-Type Ca^{2+} Channel Inhibition Do not Affect CH-Induced Myogenic Tone**

Pretreatment with diltiazem had no significant effect on myogenic tone or VSM [Ca^{2+}i], in endothelium-disrupted arteries (150–200 μm ID) from either control or CH rats (Fig. 8, A–D), suggesting a lack of involvement of L-type Ca^{2+} channels in myogenic reactivity following CH. The T-type Ca^{2+}
channel inhibitor, mibefradil (10 µM), was similarly without effect on myogenic tone in CH arteries [mibefradil (n = 4): 5 mmHg = 0.39% ± 1.61%, 12 mmHg = 8.20% ± 1.50%, 15 mmHg = 10.12% ± 0.53%, 25 mmHg = 10.39% ± 1.71%, 35 mmHg = 6.95% ± 0.72%, and 45 mmHg = 2.00% ± 0.68%; vehicle (n = 5): 5 mmHg = −3.45% ± 2.63%, 12 mmHg = 9.63% ± 1.53%, 15 mmHg = 13.13% ± 1.36%, 25 mmHg = 10.51% ± 1.40%, 35 mmHg = 5.05% ± 0.53%, and 45 mmHg = 0.69% ± 0.28%].

DISCUSSION

The major findings of this study are: 1) a Ca\textsuperscript{2+} sensitization mechanism involving ROK, but not PKC, contributes to basal VSM tone in small pulmonary arteries following CH; 2) CH induces myogenic constriction in pulmonary arteries ranging from 50 to 200 µm ID, whereas myogenicity is not observed in larger arteries; 3) myogenic reactivity in CH arteries is mediated via ROK-dependent VSM Ca\textsuperscript{2+} sensitization; and 4) PKC,
L-type, and T-type Ca\(^{2+}\) channels do not contribute to pressure-induced tone in this setting. Collectively, these data suggest that CH elicits myogenic tone in small pulmonary arteries through a mechanism involving ROK-dependent myofilament Ca\(^{2+}\) sensitization.

ROK signaling mechanisms are activated in the pulmonary vasculature following exposure to CH (17, 40, 41, 64). For example, a recent study showed that chronic administration of the selective ROK inhibitor, Y-27632, reduced pulmonary hypertension and vascular remodeling in mice exposed to hypoxia for 2 wk (17). In addition, acute intravenous infusion of Y-27632 reduced baseline pulmonary arterial pressure and vascular resistance in conscious CH rats that had been acutely returned to normoxia (41). Interestingly, the highest dose of Y-27632 normalized total pulmonary resistance between control and CH rats, suggesting that vasoconstrictor mechanisms involving ROK are of greater importance in mediating CH-induced pulmonary hypertension in rats than fixed components of hypertension, i.e., arterial remodeling and polycythemia.

Consistent with VSM Ca\(^{2+}\) sensitization contributing to elevated basal pulmonary arterial tone following CH, our present results have demonstrated an effect of NO to reduce basal tone in isolated, endothelium-disrupted pulmonary arteries from CH rats but not controls independent of a change in resting VSM [Ca\(^{2+}\)]\(_i\). This elevated VSM tone in CH arteries is not due to increases in VSM [Ca\(^{2+}\)]\(_i\), since our current observations as well as previous studies from our laboratory have consistently indicated no differences in resting VSM [Ca\(^{2+}\)]\(_i\) between control and CH arteries (24–26, 42). Considering our earlier findings that CH mediates a shift in NO signaling to mechanisms involving PKG-dependent inhibition of RhoA/ROK-induced VSM Ca\(^{2+}\) sensitization (25), we examined the hypothesis that elevated basal tone in CH arteries is dependent on ROK activity. In agreement with this possibility, we found that the selective ROK inhibitor, HA-1077, induced a concentration-dependent reduction in basal tone only in arteries from CH rats without altering VSM [Ca\(^{2+}\)]\(_i\), thus supporting a major contribution of the RhoA/ROK pathway to basal tone in the hypertensive pulmonary circulation. These data further complement previous findings from our group that both basal RhoA activity and ROK expression are elevated in small pulmonary arteries following CH (25). In contrast to effects of CH to augment ROK-dependent vasoconstriction in the pulmonary circulation, Wardle and colleagues (63) recently reported that acute hypoxia inhibits ROK-induced Ca\(^{2+}\) sensitization in porcine coronary arteries, a response that may contribute substantially to hypoxic vasodilation in the coronary circulation. Although the mechanism by which hypoxia inhibits ROK activity in coronary VSM remains to be defined, these observations, together with those of the present study, further underscore the importance of ROK signaling in mediating the

Fig. 6. Myogenic tone (% of passive ID; A and C) and VSM [Ca\(^{2+}\)]\(_i\) (B and D) as a function of intraluminal pressure in endothelium-disrupted pulmonary arteries (mean ID = 172 ± 12.5 μm) from control (A and B) and CH (C and D) rats in the presence of HA-1077 (10 μM; n = 5/group) or saline vehicle (n = 5/group). Values are means ± SE. *P < 0.05 vs. Control + Vehicle; #P < 0.05 vs. 5 mmHg intraluminal pressure within CH group. τP < 0.05 vs. CH + Vehicle. No significant pressure-induced tone was observed in Control, Control + HA-1077, or CH + HA-1077 groups.
divergent responses to hypoxia between the systemic and pulmonary circulations.

In support of the observations from the current study, Nagaoka et al. (41) have reported an effect of the ROK inhibitor Y-27632 to reduce resting tension and contractile responses to depolarizing concentrations of KCl in endothelium-intact pulmonary arterial rings from CH rats. Additional evidence supports a contribution of ROK to enhanced receptor-mediated vasoconstriction in lungs from pulmonary hypertensive animals. For example, a recent study from our laboratory (25) indicates that ROK inhibition abolishes augmented UTP-induced constriction in pressurized, Ca\textsuperscript{2+}/H\textsuperscript{11001}\textsuperscript{-permeabilized small pulmonary arteries from CH rats. Barman (4) has further demonstrated that increased endothelin-1-induced contraction in pulmonary arterial rings from CH Fawn-Hooded rats is mediated in part by ROK, although enhanced contractility to KCl was found to be unaltered by Y-27632 in hypertensive arteries. A similar contribution of ROK to endothelin-1-mediated contraction in endothelium-disrupted, intrapulmonary artery rings from CH rats has been provided by Weigand and colleagues (64). In contrast to the findings of the current study, however, baseline tension in these arteries was unaffected by treatment with Y-27632 or HA-1077 (64). The reason for these apparent discrepancies between the two studies is unclear, but it may be a consequence of the different preparations, rat strains, or vessel sizes studied.

Our present finding that PKC does not contribute to elevated basal pulmonary arterial tone or myogenic reactivity following CH is consistent with previous observations that the general PKC inhibitors GF109203X and staurosporine do not affect baseline tension in rat pulmonary arterial rings (64). Since it has been reported that higher concentrations of ROK inhibitors can inhibit PKC signaling (9), we additionally verified that the 10\textsuperscript{-5} M concentration of HA-1077 used in the current study does not affect PKC-induced constriction in pressurized small pulmonary arteries. Therefore, it appears that CH-induced elevations in basal pulmonary arterial tone and myogenic reactivity are not a function of PKC-dependent Ca\textsuperscript{2+} sensitization.

Although myogenic reactivity contributes to resting vascular tone and autoregulation of blood flow in both the systemic (8, 12, 16, 18, 38) and fetal pulmonary circulations (5, 55, 56), there is little evidence to support a contribution of myogenicity to regulation of vascular tone in the adult pulmonary circulation (5, 28). However, it is possible that distal extension of VSM into small pulmonary arteries or an alteration in VSM phenotype associated with CH provides sufficient muscularity or sensitivity to changes in stretch (54) to impart myogenic

![Fig. 7. Myogenic tone (% of passive ID; A and C) and VSM \[Ca^{2+}\]](B and D) as a function of intraluminal pressure in endothelium-disrupted pulmonary arteries (mean ID = 169.8 ± 6.1 μm) from control (A and B) and CH (C and D) rats in the presence of GF109203X (1 μM; n = 4/group) or saline vehicle (n = 5/group). Values are means ± SE. *P < 0.05 vs. respective control groups; #P < 0.05 vs. 5 mmHg intraluminal pressure within CH + vehicle and CH + GF109203X groups. No significant differences were observed between vehicle and GF109203X treatments in either control or CH groups. No significant pressure-induced tone was observed in control arteries.
behavior. Thus we hypothesized that elevated basal pulmonary arterial tone following CH is due to the development of myogenic reactivity. Consistent with this possibility, we have demonstrated pressure-dependent increases in VSM tone in small pulmonary arteries from CH rats without a corresponding change in VSM $[\text{Ca}^{2+}]/\text{H}^{+}$. Furthermore, similar to effects of ROK inhibition on basal pulmonary arterial tone discussed above, myogenic tone in CH arteries was abolished by pre-treatment with HA-1077. These findings demonstrate a novel effect of CH to induce myogenic reactivity in the pulmonary circulation and further implicate a major role for a ROK-dependent VSM $[\text{Ca}^{2+}]/\text{H}^{+}$ sensitization mechanism in mediating this response.

Interestingly, myogenic tone in CH pulmonary vessels appears to increase as a function of decreasing arterial diameter, which may explain our previous observations that slightly larger arteries from these animals do not demonstrate myogenic vasoconstriction despite pressure-dependent VSM membrane depolarization (42). These findings are consistent with previous studies that have demonstrated heterogeneity along the systemic vascular bed, with greater myogenic tone being observed in more distal segments (10, 11). We also observed no effect of L-type or T-type $\text{Ca}^{2+}$ channel blockade on CH-induced myogenic reactivity or VSM $[\text{Ca}^{2+}]$. This suggests that membrane potential does not achieve a threshold sufficient for activation of these voltage-gated $\text{Ca}^{2+}$ channels. These data stand in marked contrast to many studies of the systemic circulation and the fetal/neonatal pulmonary circulations that demonstrate a central role for $\text{Ca}^{2+}$ influx through L-channels in myogenic reactivity (12, 19, 21–23, 31). Nevertheless, it is possible that membrane depolarization-induced activation of voltage-gated $\text{Ca}^{2+}$ channels contributes to myogenic vasoconstriction in the hypertensive adult pulmonary circulation in vivo where stimuli such as hypoxia or paracrine/endocrine factors may provide additional depolarization.

It remains to be determined through what distal signaling pathways ROK mediates elevated pulmonary arterial tone following CH. Although MLCP is a primary target for ROK-dependent VSM $[\text{Ca}^{2+}]$ sensitization (53), an alternative possibility is that increases in RhoA or ROK activity augment contractility independent of MLC phosphorylation. Indeed, both RhoA and ROK can promote actin polymerization (53), which may represent an alternative mechanism of $\text{Ca}^{2+}$-independent VSM contraction involving regulation of the actin thin filament. Furthermore, recent studies suggest that ROK contributes to pressure-induced actin polymerization in the systemic vasculature (2, 8). It is also possible that ROK mediates MLC phosphorylation independent of regulatory influences on MLCP. In support of this hypothesis are in vitro MLC phosphorylation studies indicating an effect of ROK to phosphorylate MLC at the same residue (Ser19) that is phosphorylated.

Fig. 8. Myogenic tone (% of passive ID; A and B) and VSM $[\text{Ca}^{2+}]/\text{H}^{+}$ (C and D) as a function of intraluminal pressure in endothelium-disrupted pulmonary arteries (mean ID = 167.2 ± 9.3 μm) from control (A and B) and CH (C and D) rats in the presence of the L-type $\text{Ca}^{2+}$ channel inhibitor diltiazem (50 μM; n = 5/group) or saline vehicle (n = 5/group). Values are means ± SE. *P < 0.05 vs. respective control groups; #P < 0.05 vs. 5 mmHg intraluminal pressure within CH + vehicle and CH + diltiazem groups. No significant differences were observed between vehicle and diltiazem treatments in either control or CH groups. No significant pressure-induced tone was observed in control arteries.
by MLC kinase (MLCK) to increase myosin ATPase activity and induce cross-bridge cycling (3, 27, 29, 39, 43, 53). However, the functional significance of this event remains to be established. Studies in gastrointestinal smooth muscle have further implicated a role for ROK to activate a zipper-interacting protein (ZIP) kinase that phosphorylates MLC at both Ser19 and Thr18 (7, 39). Therefore, ROK may function as a Ca\(^{2+}\)-independent MLCK either directly or indirectly through activation of ZIP kinase.

An additional challenge is to establish the mechanism by which CH facilitates stretch-induced activation of the RhoA/ROK signaling pathway in pulmonary VSM. RhoA stimulation in VSM can occur secondary to activation of G protein-coupled receptors by endothelium-derived factors or alternatively to activation of ZIP kinase.

**REFERENCES**


