**RhoA Activation by Hypoxia in Pulmonary Arterial Smooth Muscle Cells Is Age and Site Specific**

Karine Bailly, Anne J. Ridley, Susan M. Hall, Sheila G. Haworth

**Abstract**—Hypoxia induces vasoconstriction of pulmonary arteries through contraction of smooth muscle cells (SMCs). The GTPase RhoA regulates smooth muscle contractility and actin cytoskeletal remodeling through the Rho-associated kinase (ROCK). We previously found that the postnatal fall in pulmonary vascular resistance was associated with actin cytoskeletal remodeling in porcine pulmonary arterial SMCs (PASMCs) in vivo. Here, we investigated the effects of acute and chronic hypoxia on the morphology and RhoA activity of PASMCs from fetal and neonatal piglets. Acute hypoxia enhanced actin stress fiber formation and RhoA activity in both inner and outer medial PASMCs from the fetus but only in the inner medial PASMCs from normal 3-day-old piglets. The increased stress fiber formation was dependent on Rho and ROCK. In outer medial PASMCs from 14-day-old animals, acute hypoxia decreased RhoA activity. Interestingly, outer medial PASMCs from animals exposed to chronic hypoxia had fewer stress fibers associated with a lower basal RhoA activity. Treatment of PASMCs from normal 3-day-old piglets with Rho or ROCK inhibitors for 24 hours induced a similar morphology. Rac activity was not altered by either acute or chronic hypoxia. These data show that acute hypoxia induces RhoA activation only in PASMCs from young animals, whereas chronic hypoxia selectively downregulates RhoA activity in outer medial PASMCs leading to an altered phenotype. *(Circ Res. 2004;94:1383-1391.)*

**Key Words:** pulmonary • smooth muscle cell • hypoxia • Rho • Rac

**P**ulmonary vascular resistance is high in utero and normally falls at birth when pulmonary arteries dilate and blood flow increases. Postnatal dilatation is characterized by elongation and thinning of medial pulmonary arterial smooth muscle cells (PASMCs) with associated actin cytoskeletal remodeling, transient disassembly of the actin cytoskeleton, and a reduction in total actin content.1,2 Cytoskeletal change is most marked in inner medial SMCs. Failure to adapt normally to extraterine life causes persistent pulmonary hypertension of the newborn (PPHN). The pulmonary arteries remain undilated and thick walled.3,4 Hypoxia is a common cause of PPHN in human infants, and newborn piglets exposed to chronic hypoxia develop pulmonary hypertension and show pulmonary vascular abnormalities similar to those seen in sick infants.5,6 Actin cytoskeleton remodeling is impaired in the PASMCs of chronically hypoxic newborn piglets, although the normal postnatal increase in proportion of monomer actin does take place.2 These observations suggest that chronic hypoxia causes disregulation of actin filament turnover in the newborn.

Rho-family GTPases are involved in actin remodeling: Rho regulates the formation of stress fibers and focal adhesions,7,8 whereas Rac induces lamellipodia and membrane ruffles.9–11 Rho cycles between a GDP-bound inactive state and a GTP-bound active state, and when bound to GTP, activates its downstream targets, including the serine/threonine kinase Rho-associated kinase (ROCK).12 The Rho-ROCK pathway can be inhibited by treating cells with either botulinum C3 exoenzyme, which ADP-ribosylates and inhibits Rho,13–15 or the ROCK inhibitor Y-27632.16 Rho has been implicated in the pathogenesis of experimental hypoxic pulmonary hypertension and systemic hypertension in the adult. Inhibition of ROCK prevents sustained hypoxic vasoconstriction in isolated rat intrapulmonary arteries and the perfused rat lung17 and the Rho-ROCK pathway is involved in the pathogenesis of increased systemic hypertension in rats, pigs, and humans.16,18,19 A recent study showed that acute hypoxia inhibits myosin phosphatase (a target of ROCK) in cultured adult rat PASMCs. The findings suggested that inhibition of myosin phosphatase is linked to activation of ROCK.20 In this article, we sought to explain the postnatal changes described in the PASMCs of intact porcine intrapulmonary arteries in vivo by studying the relationship between RhoA activity and PASMC morphology in vitro. We investigated the effect of hypoxia on RhoA activity and cell morphology of PASMCs from fetal and neonatal piglets. RhoA levels in cultured PASMCs increase with the age of the animal from which they were derived, and conversely, RhoA activation in response to

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acute hypoxia diminishes with age. Chronic hypoxia reduces RhoA activity in outer medial PASMCs associated with a decrease in stress fibers and altered morphology, which can be mimicked in normal PASMCs by treatment with Rho/ROCK inhibitors. These data indicate that there are stable changes in the differentiation state of PASMCs derived from different ages and hypoxia states, which correlate with changes in the responsiveness of RhoA to hypoxia.

Materials and Methods

Reagents
Elastase was obtained from Worthington; collagenase and trypsin inhibitor were obtained from Sigma; minimum essential medium (MEM) vitamin was obtained from Life Technologies; DAKO serum-free protein blocker was obtained from DAKO; rhodamine-phalloidin and rhodamine-labeled phalloidin, mouse anti-RhoA antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, Calif); mouse anti-Rac antibody was obtained from Upstate Biotechnology (Milton Keynes, UK); C3 exoenzyme was obtained from List Biological Laboratories; and Y-27632 was generously provided by Welfide (Osaka, Japan).

Primary Isolation of SMCs From Pulmonary Arteries
Piglets from Large White sows were killed just before term (fetal), and at 3 and 14 days of age. One group of piglets was exposed to chronic hypobaric hypoxia from 3 to 14 days of life. Each group consisted of animals taken from at least 3 different litters, and all but the hypoxic animals were sexually fed. During exposure to hypoxia, the piglets were fed mashed feed and milk. In the hypobaric chamber, the internal temperature (25°C) and light were controlled, and the air pressure was maintained at 50.8 kPa. Hypoxic animals were killed immediately after removal from the hypobaric chamber. The animals received humane care in compliance with British Home Office Regulations and with the Principles of Laboratory Animal Care formulated by the National Society of Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Science and published by the National Institutes of Health (NIH publication 80 to 23, revised 1985). Tissue taken from piglet lungs was treated within 1 hour of death. In each animal, the elastic conduit intrapulmonary artery distal to the origin of the upper lobe branch was dissected (lower lobe pulmonary artery), the proximal half of this vessel was cut open, and the endothelium was removed by gentle scraping of the luminal surface of the vessel with a scalpel blade. The tunica media was then mechanically dissected into 2 layers: a subendothelial inner medial layer and an outer medial layer. Tissue explants from each medial layer were incubated in dissociation medium (HEPES-buffered basal salt solution; Life Technologies) containing 0.5 mg/mL elastase, 15 mg/mL BSA, 100 µL of MEM vitamin) for 45 minutes at 37°C with constant agitation. Cells were then washed twice in RPMI medium 1640 (PAA Laboratories) with 10% fetal calf serum (PAA Laboratories) and expanded in the same medium. All experiments were performed on cells at passages 3 through 7.

Exposure of PASMCs to Acute Hypoxia
Cells were plated and grown for 7 to 10 days to reach subconfluence. After serum deprivation for 24 hours, PASMCs were transferred into an incubator (Wolf Laboratories), flushed with 5% O2, 5% CO2, and 90% N2 for 1 to 8 hours. Some PASMCs from normal 3-day-old animals were exposed to hypoxia for 24 hours.

Immunofluorescence and Confocal Laser Scanning Microscopy
Cells on coverslips were washed in PBS (Sigma) and fixed in 4% paraformaldehyde and 2% sucrose in PBS for 20 minutes at room temperature, permeabilized with 0.1% Triton X-100 in PBS, and treated with blocking solution (DAKO serum-free protein blocker) for 30 minutes. Cells were stained with rhodamine-labeled phalloidin (1 µg/mL) for 20 minutes at room temperature to localize actin filaments, then washed and mounted on slides with DPX mountant (Merck). Labeled cells were examined under a Radiance 2000 confocal microscope (Bio-Rad) using a 20X water immersion objective (Olympus). Specimens were illuminated with 21% nonsaturating levels of the 543 nm line of a helium neon laser, and emissions were filtered with a 590 emission filter. Images of consecutive, 2.6-µm-thick optical sections were obtained and projection performed using Lasersharp software.

Affinity Precipitation of GTP-Rho and GTP-Rac
The expression vectors pGEX-2T-Rho-binding domain (RBD), encoding glutathione S-transferase (GST)-Rhotekin RBD, and pGEX-2T-Rac-binding domain (PBD), encoding GST-p21-activated kinase PBD, were kind gifts from Martin Schwartz ( Scripps Institute, La Jolla, Calif) and John Collard (Amsterdam, The Netherlands), respectively. GST-RBD and GST-PBD were purified as described previously. Immediately after exposure to acute hypoxia, PASMCs were washed twice with ice-cold PBS, and affinity precipitation of either GTP-Rho or GTP-Rac was performed with GST-RBD or GST-PBD as described previously. Bound RhoA or Rac proteins were detected by Western blotting using monoclonal antibodies against RhoA and Rac. In each experiment, to confirm that equal amounts of either RhoA or Rac protein were expressed, samples of the starting lysate were immunoblotted with either the anti-RhoA or anti-Rac antibody.

Treatment With C3 Exoenzyme and Y-27632 Inhibitor
After serum deprivation, inner and outer medial PASMCs from 3-day-old normal animals were exposed to acute hypoxia for 8 hours and were incubated with either 20 µg/mL of C3 exoenzyme (RhoA inhibitor) during the whole 8-hour period, or with Y-27632 (ROCK inhibitor) for 1 hour starting at 7 hours after the initiation of hypoxic exposure. The longer incubation with C3 exoenzyme was required because it is not very cell permeable. Alternatively, after serum deprivation for 24 hours, PASMCs were incubated for 24 hours in normoxic conditions with either 20 µg/mL of C3 exoenzyme or 10 µmol/L Y-27632.

Statistical Analysis
Student t test was used to compare results between experimental groups. Results were expressed as mean values ± SE. A value of P<0.05 was considered statistically significant.

Results
RhoA Protein Levels in PASMCs Increase With Age of Animals
In the basal unstimulated state, the morphology of PASMCs derived from animals of different ages was similar and all had stress fibers, although the outer medial PASMCs from 3- and 14-day-old piglets appeared to have more stress fibers than other cells ( Figure 1, control panels). RhoA expression in PASMCs from both inner and outer media of fetal piglets was very low and increased progressively in 3- and 14-day-old piglets (Figure 2D).

PASMCs Display Age-Related and Site-Specific Differences in Hypoxia-Induced Stress Fiber Formation and RhoA Activation
Exposure to low oxygen tension for 8 hours induced stress fiber formation in PASMCs from both the inner and outer layers of the media of fetal piglets, only in the cells from the
inner media of 3-day-old piglets, and not in cells from either inner or outer media of 14-day-old animals (Figure 1).

To investigate whether these actin cytoskeletal changes were regulated by RhoA, RhoA activity was determined by affinity precipitation with the GST-RBD.22 Acute hypoxia enhanced RhoA activity in inner and outer medial PASMCs from fetal piglets between 2 and 8 hours of hypoxic exposure (Figure 2A). In PASMCs from 3-day-old piglets, RhoA activity increased progressively in the inner media during the 8-hour exposure period but did not change in the outer medial cells (Figure 2B). In PASMCs from 14-day-old piglets, acute hypoxia did not enhance RhoA activity in either inner or outer medial PASMCs. Rather, the PASMCs from the outer media showed a decrease in RhoA activity after a 1-hour exposure to acute hypoxia (Figure 2C). In all cases, after 16 hours of exposure to hypoxia, RhoA activity was similar to the basal level in serum-starved cells (data not shown).

In contrast to RhoA, acute hypoxia did not affect the level of Rac activity24 in either inner or outer medial PASMCs at any age (Figure 3A through 3C). These results indicate that PASMCs from the inner media of 3-day-old animals show the strongest response to acute hypoxia in terms of RhoA activation, and hypoxia-induced stress fiber formation correlates with RhoA activation.

**Acute Hypoxia-Induced Stress Fiber Formation Involves Rho and ROCK Activation**

To examine the involvement of either Rho or ROCK in acute hypoxia-induced stress fiber formation, PASMCs from 3-day-old piglets were treated with either C3 exoenzyme, which specifically inhibits the function of Rho, or Y-27632, a specific inhibitor of ROCK. In the absence of either C3 exoenzyme or Y-27632, as expected, acute hypoxia induced stress fiber formation in PASMCs from the inner but not the outer media (Figure 4). Treatment with both C3 exoenzyme and Y-27632 during hypoxic exposure induced disassembly of stress fibers in the inner but not outer medial PASMCs. These results suggest that actin cytoskeleton remodeling induced by acute hypoxia involves Rho-ROCK pathway activation. However, basal levels of stress fibers are not dependent on Rho-ROCK signaling in outer medial PASMCs, which do not activate RhoA in response to acute hypoxia.

**PASMCs From Chronically Hypoxic Piglets Show Altered RhoA Activity and Actin Cytoskeletal Organization**

To determine whether chronic exposure to hypoxia affects cell morphology or RhoA activity, PASMCs were isolated from 14-day-old piglets that had been exposed to hypoxia for 11 days. In the basal unstimulated state, the appearance of the inner medial PASMCs from 14-day-old chronically hypoxic piglets was similar to that of cells from the inner media of normal animals of the same age (Figure 5). In contrast, chronic hypoxia induced a morphological change of the outer medial PASMCs: the cells were less elongated with fewer stress fibers and more areas of punctate F-actin than the outer medial PASMCs from normal piglets (Figure 5). This correlated with a decrease in basal RhoA activity in the outer medial PASMCs compared with normal piglets, but no change in the inner medial cells (Figure 6C). There was no significant difference in Rac activity between normal and hypoxic piglets in either inner or outer medial PASMCs (data not shown).

**Effects of Acute Hypoxia on PASMCs From Chronically Hypoxic Piglets**

Acute hypoxia did not change the appearance of the cytoskeleton in either inner or outer medial PASMCs of the chronically hypoxic 14-day-old animals (Figure 5), and RhoA activity did not change in inner medial PASMCs. These findings are similar to those in cells from normal 14-day-old animals. RhoA activity remained unchanged in the outer medial PASMCs, however, unlike the cells from 14-day-old normal piglets, which showed a decrease in activity (Figures 6A and 2C). As in cells from normal animals, acute hypoxia did not alter Rac activity in either inner or outer medial PASMCs from chronically hypoxic piglets (Figures 6B and 3C).
In an attempt to produce in vitro the abnormal morphological phenotype produced in vivo by exposure to chronic hypoxia, we exposed PASMCs from 3-day-old normal animals to prolonged hypoxia and found that the abnormal phenotype could be reproduced in vitro after 24-hour exposure but only in cells derived from the outer media and not the inner media (Figure 7A). This result suggests that chronic hypoxia preferentially promotes morphological change in outer medial PASMCs.

Because RhoA activity was decreased in outer medial PASMCs from chronically hypoxic compared with normal animals, we next tested whether specific inhibition of the Rho-ROCK pathway by the Rho inhibitor C3 exoenzyme or the ROCK inhibitor Y-27632 would induce the development of the abnormal phenotype. Treatment of serum-starved inner medial PASMCs from 3-day-old piglets with C3 exoenzyme or Y-27632 for 24 hours resulted in a significant reduction of stress fibers and an increase in punctate F-actin (Figure 7B), a morphology similar to that of the outer medial PASMCs from chronically hypoxic piglets. There was no difference in response between inner and outer medial PASMCs derived from fetal, 3-, and 14-day-old piglets were immunoblotted with anti-RhoA antibody. Equal amounts of protein were loaded in each lane.

Discussion

The present study shows that acute hypoxia enhanced stress fiber formation through activation of the Rho-ROCK path-
Figure 3. Regulation of Rac activity by acute hypoxia. Some serum-starved PASMCs from the inner or outer media of fetal (A), 3-day old (B), and 14-day-old (C) piglets were incubated in a low O₂ concentration for varying periods of time. Top immunoblots show activated GTP-bound Rac; middle panels show the amount of Rac protein in whole-cell lysates. The amount of activated Rac was normalized to the amount of Rac in whole-cell lysates. The histograms show the results of densitometric analysis of a representative experiment from 3 separate experiments.
way in PASMCs from young animals. In contrast, Rac activity was not influenced by exposure to acute hypoxia in cells from either normal or chronically hypoxic animals. We also report that chronic hypoxia induced morphological change only in the outer medial PASMCs. Fewer stress fibers were formed, a change that appeared to be caused by a decrease in basal RhoA activity. Exposure to chronic hypoxia inhibited the reduction in RhoA activity seen in normal outer medial PASMCs on exposure to acute hypoxia.

Actin Cytoskeletal Regulation by RhoA in the Acute Hypoxia Response of PASMCs

In PASMCs from young animals, acute hypoxia induced actin cytoskeleton remodeling associated with Rho activation. These changes were abolished by inhibition of the Rho-ROCK pathway, suggesting a cause-and-effect relationship. These results are in accord with previous studies showing that in human airway SMCs, lysophosphatidic acid-induced, endothelin-1-induced, and carbachol-induced actin reorganization involved the activation of Rho.25,26 Rho-ROCK activity is known to increase the reactivity of pulmonary arteries by increasing their amplitude of contraction and reducing their response time,27–29 and to enhance SMC contractility by increasing myosin light chain (MLC) phosphorylation.8 Acute hypoxia induces pulmonary vasoconstriction30 as an adaptive response of the pulmonary circulation to regional differences in alveolar oxygen tension. Local vasoconstriction in response to alveolar hypoxia leads to redirection of blood flow to areas of the lungs with higher oxygen tension. Inhibition of ROCK prevents sustained hypoxic vasoconstriction in the isolated intrapulmonary arteries and perfused rat lung suggesting that ROCK may be a pivotal step in the generation of sustained hypoxia-induced pulmonary vasoconstriction (HPV).17 In rat PASMCs, acute hypoxia activates ROCK, inhibits myosin phosphatase, and induces MLC phosphorylation.20,31 In the present study, we demonstrated Rho-dependent cytoskeleton remodeling in response to acute hypoxia in PASMCs, confirming the importance of the Rho-ROCK pathway in effecting acute pulmonary vasoconstriction.

It appears that during development, PASMCs progressively lose their ability to activate RhoA in response to acute hypoxia. This could be related to changing patterns of gene expression during development:32–36 for example, smooth muscle myosin heavy chain expression transcriptional level is greatest in SMCs from newborn animals and decreased in older animals, consistent with their greater degree of differentiation.33,35 However, it is well known that the adult lung vasoconstricts in response to acute hypoxia. On the basis of our results, we propose that RhoA contributes to acute hypoxia-induced vasoconstriction in neonatal piglets, but other mechanisms for stimulating smooth muscle contraction, for example RhoA-independent ROCK activation37 or MLC kinase activation, predominate in older animals.

Moreover, in PASMCs from 3-day-old animals, acute hypoxia-induced stress fiber formation and increased RhoA activity were only observed in cells from the inner media. Other investigators have demonstrated that the medial layer is made up of several different subpopulations of SMCs, which exhibit differences in proliferative and matrix-producing
responses to a hypoxic environment. Our observations of differences in RhoA activation after exposure to acute hypoxia emphasize the fact that there are intrinsic functional differences between PASMCs, depending on the age of the animal and on their proximity to the lumen of the arterial vessel. Interestingly, acute hypoxia did not induce any activation of Rac. In contrast, in epithelial cells, Rac1 is activated in response to acute hypoxia and plays an essential role in hypoxia-inducible factor protein expression and transcriptional activity.

**Effects of Chronic Hypoxia on PASMC Morphology and Responses to Acute Hypoxia**

Chronic hypoxia of 11-day duration completely altered the actin cytoskeleton of the outer but not inner medial PASMCs and reduced basal RhoA activity. It is noteworthy that the morphology of the cells was stable and did not revert to a normal phenotype in culture, where conventionally, the oxygen tension in the incubator is higher. In addition, inhibiting the Rho-ROCK pathway in normal cells mimicked the changes in shape induced by chronic hypoxia. A possible explanation for the altered shape is that there is a shift in the balance between Rho and Rac activity because Rac activity is not altered by chronic hypoxia. Increased Rac activity in fibroblasts and neuroblastoma cells correlates with a more epithelial morphology. Decreased RhoA activity and less stress fibers could also affect SMC differentiation because RhoA-dependent regulation of the actin cytoskeleton selectively regulates SMC differentiation marker gene expression by modulating serum response factor (SRF)-dependent transcription. Recently, Liu et al demonstrated that the Rho-ROCK pathway controls smooth muscle gene transcription in airway myocytes, in part through regulating the nuclear localization of SRF.

Chronic hypoxia leads to pulmonary hypertension in piglets, and systemic hypertension induced in adult rats has been shown to correlate with an increase in RhoA activity in aortic SMCs. On the other hand, exposure of adult rats to chronic hypoxia led to a decrease in RhoA expression in the pulmonary artery. Together with our observations that PASMCs from chronic hypoxic hypertensive piglets have unchanged (inner) or markedly decreased (outer) basal RhoA

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**Figure 6.** Regulation of RhoA and Rac activities in chronic hypoxic piglets. After serum starvation for 24 hours, some inner and outer PASMCs from 14-day-old piglets that had been exposed to chronic hypoxia from 3 to 14 days of age were incubated in a low O2 concentration for varying periods of time. RhoA (A) and Rac (B) activities were determined as described in Figures 2 and 3. C, Basal RhoA activities of serum-starved inner or outer medial PASMCs from normal and chronically hypoxic piglets in normoxic conditions were assessed. The Western blot shows a representative experiment from 3 separate experiments. In the histogram, RhoA activities were normalized to the amount of RhoA in whole-cell lysates.
activity, these results suggest that RhoA activity is differentially affected in hypertension, depending on the stimulus and/or the circulation in question.

It is interesting that only the outer medial PASMCs showed an altered morphology after chronic hypoxia. We also observed a functional difference between inner and outer medial PASMCs with regard to the effect of chronic hypoxia on their responses to acute hypoxia. Indeed, chronic hypoxic exposure for 11 days in vivo did not alter the effects of acute hypoxia on stress fiber formation or RhoA and Rac activities in inner medial PASMCs, but in the outer medial PASMCs, chronic hypoxia prevented the decrease in RhoA activity seen in RhoA activity in normal cells. The influence of chronic hypoxia on acute HPV may depend both on the species of animal studied and the localization of the PASMCs within the media. These results emphasize regional differences in PASMC populations within the media.

Conclusions

In conclusion, we have demonstrated for the first time that in PASMCs, acute hypoxia can induce activation of RhoA but not Rac, leading to actin cytoskeleton remodeling, and that these responses display regional and age-related differences. Moreover, we showed that PASMCs from chronically hypoxic pulmonary hypertensive piglets undergo a morphological change related to decreased basal RhoA activity. In the future, it will be important to study peripheral pulmonary resistance arteries, investigate the factors (gene expression and/or environmental cues) responsible for this heterogeneity in PASMC responses during early life, and to define the effectors upstream of RhoA that link its activation to the hypoxic response in PASMCs.

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References

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