Role of RhoB in the Regulation of Pulmonary Endothelial and Smooth Muscle Cell Responses to Hypoxia

Beata Wojciak-Stothard, Lan Zhao, Eduardo Oliver, Olivier Dubois, Yixing Wu, Dimitris Kardassis, Eleftheria Vasilaki, Minzhou Huang, Jane A. Mitchell, Louise S. Harrington, George C. Prendergast, Martin R. Wilkins

Rationale: RhoA and Rho kinase contribute to pulmonary vasoconstriction and vascular remodeling in pulmonary hypertension. RhoB, a protein homologous to RhoA and activated by hypoxia, regulates neoplastic growth and vasoconstriction but its role in the regulation of pulmonary vascular function is not known.

Objective: To determine the role of RhoB in pulmonary endothelial and smooth muscle cell responses to hypoxia and in pulmonary vascular remodeling in chronic hypoxia-induced pulmonary hypertension.

Methods and Results: Hypoxia increased expression and activity of RhoB in human pulmonary artery endothelial and smooth muscle cells, coincidental with activation of RhoA. Hypoxia or adenosinergic overexpression of constitutively activated RhoB increased actomyosin contractility, induced endothelial permeability, and promoted cell growth; dominant negative RhoB or manumycin, a farnesyltransferase inhibitor that targets the vascular function of RhoB, inhibited the effects of hypoxia. Coordinated activation of RhoA and RhoB maximized the hypoxia-induced stress fiber formation caused by RhoB/mammalian homolog of Drosophila diaphanous-induced actin polymerization and RhoA/Rho kinase-induced phosphorylation of myosin light chain on Ser19. Notably, RhoB was specifically required for hypoxia-induced factor-1α stabilization and for hypoxia- and platelet-derived growth factor-induced cell proliferation and migration. RhoB deficiency in mice markedly attenuated development of chronic hypoxia-induced pulmonary hypertension, despite compensatory expression of RhoA in the lung.

Conclusions: RhoB mediates adaptational changes to acute hypoxia in the vasculature, but its continual activation by chronic hypoxia can accentuate vascular remodeling to promote development of pulmonary hypertension. RhoB is a potential target for novel approaches (eg, farnesyltransferase inhibitors) aimed at regulating pulmonary vascular tone and structure. (Circ Res. 2012;110:1423-1434.)

Key Words: hypoxia ■ pulmonary hypertension ■ Rho GTPases ■ endothelium ■ smooth muscle cells

Chronic hypoxia-induced pulmonary hypertension is characterized by increased right ventricular afterload from increased pulmonary vascular resistance due to pulmonary vasoconstriction and pulmonary vascular remodeling.1 Hypoxic pulmonary vasoconstriction helps to maintain oxygen supply within physiological limits by diverting blood from poorly ventilated to better ventilated areas of the lung.2 Activation of RhoA/Rho kinase in pulmonary vasculature contributes to sustained hypoxic pulmonary vasoconstriction and pulmonary vascular remodeling in hypoxia-induced pulmonary hypertension and in other forms of pulmonary hypertension.3,4 RhoA is a member of the Rho guanosine triphosphatases (GTPases) family of proteins, key regulators of actin-dependent processes such as cell adhesion, migration and proliferation.5 In the vascular wall, activation of RhoA and Rho kinase increases smooth muscle cell contractility, increases endothelial permeability, and inhibits NO generation by decreasing endothelial nitric oxide synthase expression and activity.6–8 Inhibition of the RhoA/Rho kinase pathway contributes to the beneficial effects of sildenafil in pulmonary hypertension but statins, another inhibitor of this pathway, have little effect on the course of human pulmonary hypertension.9–11 Recent data implicate RhoB, a protein 85% homologous to RhoA and highly expressed in the lung,12 in cell responses to oxidative stress. RhoB is activated by hypoxia13,14 and geno-
vascularization has been noted. Embryonic fibroblasts from mouse embryos do not express RhoB. Genetic deletion of RhoB in mice does not affect cell survival and proliferation. Genetic deletion of RhoB in vascular smooth muscle cells also does not affect cell survival and proliferation. RhoB, like other Rho GTPases, requires prenylation for its activity but is distinguished from homologous Rho proteins by being both farnesylated and geranylgeranylated, whereas other Rho isoforms are solely geranylgeranylated. RhoB also lacks a protein kinase A/protein kinase G-specific phosphorylation site, important in the regulation of RhoA activity. Geranylgeranylated and farnesylated RhoB are thought to have different functions in cells. Farnesylated RhoB decreases hypoxia-induced cytoskeletal remodeling and induced cell apoptosis, whereas geranylgeranylated RhoB promotes cell survival and proliferation.

RhoB is a convergence point of several pathways implicated in the pathogenesis of pulmonary hypertension. RhoB acts upstream of hypoxia inducible factor (HIF)-1α and nuclear factor kappa B, mediates the effects of transforming growth factor (TGF-β) and bone morphogenetic protein on actin remodeling, and regulates trafficking of platelet-derived growth factor (PDGF) and epidermal growth factor receptors as well as nonreceptor kinases src and Akt in cells, important in the regulation of cell survival and proliferation.

Genetic deletion of RhoB in mice does not adversely affect mouse development, though retinal vascularization has been noted. Embryonic fibroblasts from RhoB null mice show increased sensitivity to TGF-β stimulation.

This study addresses for the first time the role of RhoB in the regulation of pulmonary vascular responses to hypoxia in vitro and in vivo. We show that RhoB is required for hypoxia-induced cytoskeletal remodeling, increased endothelial permeability, and associated growth responses in pulmonary vascular cells. Genetic deletion of RhoB attenuates development of chronic hypoxia-induced pulmonary hypertension in mice, likely to result from inhibition of HIF signaling and reduced pulmonary vascular remodeling.

Methods

An expanded Methods section is provided in the online-only Data Supplement.

Cell Culture

Human pulmonary artery endothelial cells (HPAECs) were cultured in endothelial growth medium-2, whereas human pulmonary artery smooth muscle cells (HPASMCs) were cultured in smooth muscle cell growth medium-2 under normoxic conditions (20% O2, 5% CO2, 95% N2) for 1 to 48 hours.

RT-PCR

Semiquantitative RT-PCR was performed with isoform-specific primers:

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>RhoA Forward</td>
<td>5'-CAGAAAAAGTGGACCCGAGAA</td>
</tr>
<tr>
<td>RhoA Reverse</td>
<td>5'-GCAGCTCTGAGCACTT</td>
</tr>
<tr>
<td>RhoB Forward</td>
<td>5'-GGGAATGGACCTTGAG</td>
</tr>
<tr>
<td>RhoB Reverse</td>
<td>5'-GGGATGaCCTTGCCACACAGCCTT</td>
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For mouse lung tissues, the primers and PCR conditions were used as in Wheeler et al.

Rho GTPases Protein Expression and Activity

RhoA and RhoB protein expression in cells and tissues was studied by immunofluorescence and Western blotting. Active RhoA and RhoB were measured with recombinant GST-RBD in GTP-loading assays.

Manipulation of RhoA/RhoB Expression and Activity in Cultured Cells

Overexpression of AdGFP (adenoviral control), dominant negative RhoB (DNRhoB; Ad-6myc-N19RhoB-GFP), constitutively activated RhoB (CARhoB; Ad-HA-V14RhoB-GFP) and dominant negative RhoA (DNRhoA; Ad-Flag-N19RhoA) was induced by adenoviral gene transfer. Farnesyltransferase inhibitor, manumycin, was added to the cells at the start of hypoxic exposure or 1 hour prior the hypoxic exposure, as indicated. Rho kinase inhibitor, Y-27632 (5 μmol/L; Calbiochem) was added to the cells overexpressing CARhoB 2 hours before cell fixation. mDia siRNA or nontargeting siRNA was introduced to cells by lipofectamine transfection and the experiments were carried out 72 hours posttransfection.

Endothelial Cell Permeability and Morphology

The effects of hypoxia and RhoB over expression on transendothelial permeability were studied using HPAECs grown in Transwell-Clear chambers. Changes in cell morphology were observed using TRITC-phalloidin labeled F-actin and immunofluorescence staining of vascular endothelial (VE)-cadherin in cells grown on cover slips.

Non-standard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>CARhoB</td>
<td>Constitutively activated RhoB</td>
</tr>
<tr>
<td>DNRhoA</td>
<td>Dominant-negative RhoA</td>
</tr>
<tr>
<td>DNRhoB</td>
<td>Dominant negative (N19)RhoB</td>
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<tr>
<td>GTPase</td>
<td>Guanosine triphosphatase</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia inducible factor</td>
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<tr>
<td>HPAEC</td>
<td>Human pulmonary arterial endothelial cell</td>
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<tr>
<td>HPASMCs</td>
<td>Human pulmonary arterial smooth muscle cells</td>
</tr>
<tr>
<td>mDia</td>
<td>Mammalian homolog of Drosophila diaphanous (mDia)</td>
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<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
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<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>VE</td>
<td>Vascular endothelium</td>
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**Cell Metabolic Activity**
An [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium colorimetric assay (Promega) was used to assess metabolic activity associated with cell proliferation and migration. PDGF-BB (20 μg/L) was added to cells in reduced serum (1% fetal calf serum) and growth factor-depleted medium and incubated for 48 hours.

**Cell Migration**
Cell migration was measured in an in vitro wound assay. HPASMC monolayer was scraped with the tip of a 100-μL pipette and the number of cells that migrated out of the wound edge during o/n incubation was scored.

**HIF Expression and Activity**
HIF activity was studied in a osteosarcoma cell line stably expressing a luciferase reporter construct under the control of a hypoxia response element (kind gift of Dr M. Ashcroft, University College London). Changes in HIF-1α protein levels in cultured HPAECs was studied by Western blotting, whereas its localization was studied by immunofluorescence and confocal microscopy.

**Apoptosis**
Apoptosis was assessed by measuring Tetramethylrhodamine, ethyl ester, perchlorate (Invitrogen) fluorescence in mitochondria of live cells using confocal microscopy and image analysis.

**Intracellular Calcium Levels**
Intracellular calcium levels in live cells were studied with Rhod3 imaging kit (Molecular Probes). Rhod3 fluorescence was measured in Glomax spectrophotometer (Promega) at excitation/emission 550/580 nm.
Normoxia and Chronic Hypoxia Studies In Vivo

All studies were conducted in accordance with UK Home Office Animals (Scientific Procedures) Act 1986 and institutional guidelines. Twelve- to 15-week-old C57BL male mice (20 g; Charles River, Margate, UK) and RhoB\(^{-/-}\)/H11002 male mice (a kind gift of Professor Brian Morris) were either housed in normal air or placed in a normobaric hypoxic chamber (FIO\(_2\) 10%) for 2 weeks (n=4–8/group). Development of pulmonary hypertension was confirmed as described previously.\(^{31}\)

Western Blot Analysis and Immunostaining

RhoA, RhoB, MLC, MLC-P–Ser19, HIF-1\(_{\alpha}\), VE-cadherin, proliferating cell nuclear antigen (PCNA), and \(\beta\)-actin protein expression was studied by Western blotting and by immunofluorescence in cells or lung lysates, as appropriate.

Statistical Analysis

All the experiments were performed in triplicate and data are presented as the mean±SEM. Comparisons between groups were carried out using 2-tailed Student \(t\) tests or 1-way ANOVA as appropriate. Statistical significance was accepted for \(P\leq0.05\) and tests were performed using GraphPad Prism version 4.0 or Microsoft Office Excel 2007.

Results

Hypoxia Increases RhoB Expression and Activity

Exposure of HPAECs and HPASMCs to hypoxia induced a 2-fold increase in RhoB gene expression, maximal between 0.5 to 2 hours of hypoxia (Figure 1A and 1B). RhoB protein levels and activity also increased significantly, reaching maximum between 2 to 4 hours (Figure 1A and 1B). Although RhoA gene and protein expression\(^{30,32}\) remained relatively unchanged, we observed a 2-fold increase in RhoA activity in both HPAECs and HPASMCs, coincident with RhoB activation (Figure 1C and 1D).

RhoB Mediates Hypoxia-Induced Cytoskeletal Remodeling in Pulmonary Vascular Cells

Hypoxia (1–24 hours) induced stress fiber formation in HPAECs and HPASMCs, and dispersion of intercellular adherens junction protein, VE-cadherin, in HPAECs (Figure 2A and 2B and Online Figure I). To examine the role of RhoB in modulating the pulmonary cell phenotype, cells were infected with adenoviruses to induce over expression of AdGFP (adenoviral control), constitutively activated RhoB (CARhoB; V14RhoB-GFP) or dominant negative RhoB (DNRhoB; N19RhoB-GFP), or were treated with manumycin (5 \(\mu\)mol/L, 2 hours incubation), a farnesyltransferase inhibitor that ablates RhoB function. AdGFP (adenoviral control) did not affect cell phenotype in normoxia or in hypoxia (Online Figure I). CARhoB increased stress fiber levels in cells and induced dispersion of intercellular adherens junctions in HPAECs in normoxia, mimicking the effects of hypoxia (Figure 2A and 2B and Online Figure I); conversely, DNRhoB and manumycin inhibited the effects of hypoxia (Figure 2A and 2B).

Changes in the localization of VE-cadherin in hypoxia were accompanied by a significant, 2-fold increase in endothelial permeability (Figure 2C). CARhoB increased endothelial permeability in normoxia (3-fold increase, comparison with normoxic control) and in hypoxia (2.4-fold increase, comparison with hypoxic control), whereas DNRhoB and manumycin preserved endothelial junctional integrity in hypoxic conditions (Figure 2C).

RhoA is highly homologous to RhoB and could compensate for changes in RhoB activity. CARhoB had no effect although DNRhoB induced a small (1.2-fold) increase in RhoA activity in cells (Online Figure II). To investigate the contribution of RhoA to hypoxia-induced morphological remodeling we overexpressed the DNRhoA (N19RhoA) alone or in combination with DNRhoB in cells. DNRhoA attenuated hypoxia-induced stress fiber formation (Online Figure III) and prevented a hypoxia-induced increase in endothelial permeability (Figure 2C), consistent with previous reports.\(^{30}\) The combination of RhoA and RhoB inhibition...
was similar to that of manumycin (Figure 2C). Manumycin inhibited RhoB activity but not RhoA, consistent with the fact that RhoB but not RhoA exists as a farnesylated isoform in vivo (Online Figure IV). We conclude that RhoA and RhoB are both important in the regulation of hypoxia-induced responses and that RhoB inhibition is not masked by a compensatory increase in RhoA activity.

**RhoA and RhoB Cooperate in Stress Fiber Formation in Pulmonary Vascular Cells**

Phosphorylation of Serine 19 in myosin light chains is required for actomyosin contraction and is promoted by hypoxia (Figure 3A and 3B). MLC phosphorylation was maximal between 2 to 4 hours of hypoxic exposure (Figure 3A and 3B) and coincided with RhoA/RhoB activation and stress fiber formation in cells. CARhoB enhanced MLC phosphorylation, partially mimicking the effects of hypoxia (1.8-fold increase in normoxia, \( P \leq 0.01 \), compared with untreated normoxic control) (Figure 3C and 3D). Inhibition of RhoB and RhoA, separately, reduced MLC phosphorylation (1.5-fold decrease and 2.5-fold decrease, respectively, compared with hypoxic control) (Figure 3C and 3D). Combined inhibition of RhoA and RhoB had a greater inhibitory effect (5.7-fold reduction compared to hypoxic control, \( P \leq 0.001 \)), suggesting that both RhoA and RhoB contribute to hypoxia-induced MLC phosphorylation of Ser19 (Figure 3C and 3D).

Of note, DNRhoB and manumycin completely abolished MLC-pSer19 localization to stress fibers (Online Figure V).

In contrast to RhoA, the effects of RhoB appear to be largely independent of Rho kinase. The putative Rho kinase inhibitor, Y-27632, had very little effect on CARhoB-induced cell morphology (Figure 4). Similarly, DNRhoA did not affect the CARhoB-induced cell phenotype in HPAECs and HPASMCs (Online Figure VI). In contrast, manumycin or knockdown of formin mDia completely abolished CARhoB-induced stress fiber formation in cells (Figure 4).

**RhoB Mediates Proliferative and Migratory Responses of Pulmonary Vascular Cells to Hypoxia**

Hypoxia induced a 1.3-fold increase in growth response in HPAECs and a greater 1.7-fold increase in HPASMCs (\( P \leq 0.05 \) and \( P \leq 0.001 \), respectively, compared with normoxic controls). The inhibitory effect of RhoB on hypoxia-induced proliferation and migration in pulmonary vascular cells was also shown in Figure 4.
RhoB is important in recycling and membrane targeting of the PDGF receptor.\textsuperscript{28,34} PDGF-BB is an important stimulator of HPASMC growth and migration in hypoxic conditions, contributing to pulmonary vascular remodeling in pulmonary hypertension.\textsuperscript{34,35} Both DNRhoA and manumycin inhibited PDGF-stimulated HPASMC growth, comparable to that caused by the PDGFR tyrosine kinase inhibitor, imatinib (Figure 6A). Conversely, imatinib inhibited hypoxia-induced RhoB expression and activation in HPASMCs (Online Figure VIII) and the effect of overexpression of RhoB on HPASMC proliferation in normoxia and hypoxia (Figure 6C). Interestingly, both activated and inhibitory RhoB mutants abrogated cell migration, suggesting that finely choreographed dynamic changes in RhoB activity are required to support continuous remodeling of actin cytoskeleton and cell adhesion during cell movement (Figure 6B and 6D and Online Figure VIII).

RhoB induces apoptosis in several types of cancer. However, in human pulmonary vascular cells, RhoB exerts a growth promoting rather than a proapoptotic effect, consistent with mouse studies documenting a function for RhoB in endothelial cell survival and angiogenesis.\textsuperscript{29} Overexpression of RhoB mutant proteins had no significant effect on cell apoptosis (Online Figure IX). Short-term (1–4 hours) incubation of cells with manumycin did not induce apoptosis (not shown) but 48 hours incubation had a proapoptotic effect (1.9-fold decrease in Tetramethylrhodamine, ethyl ester, perchlorate fluorescence, \(P<0.05\); comparison with untreated control) (Online Figure IX).

**RhoB Knockout Attenuates Development of Chronic Hypoxia-Induced Pulmonary Hypertension**

There were no significant differences in the right ventricular systolic pressure between the wild type and the RhoB\textsuperscript{−/−} mice kept in normal air (Figure 7A). Following 2 weeks of hypoxia, the right ventricular systolic pressure in wild type mice increased from 21.63±2.27 mm Hg to 36.98±1.39 mm Hg (1.7-fold increase, \(P<0.001\), compared with a normoxic group), whereas RhoB\textsuperscript{−/−} mice showed a significantly attenuated response (increase from 20.54±1.87 to 26.09±0.92 mm Hg; 1.3-fold increase, \(P<0.001\), compared with hypoxic wild type mice) (Figure 7A and 7B).

Chronic hypoxia induced right ventricular hypertrophy in both wild type and RhoB\textsuperscript{−/−} mice. This was significantly attenuated in RhoB\textsuperscript{−/−} mice (\(P<0.05\), compared with hypoxic wild type mice; Figure 7C).

Pulmonary vascular muscularization increased markedly in chronic hypoxia in both genotypes but to a significantly lesser degree in RhoB\textsuperscript{−/−} mice (79% SD 9 versus 56% SD 15, respectively, \(P<0.01\); Figure 7D and 7E).

RhoB\textsuperscript{−/−} genotype of each mouse was confirmed by PCR (Online Figure X). The effects of RhoB knockout were evident despite increases in RhoA expression. RhoB\textsuperscript{−/−} mice showed a compensatory increase in RhoA expression in basal conditions (Online Figure X), Following exposure to chronic hypoxia, the lungs from wild type mice showed increased expression of both RhoA and RhoB, whereas the lungs of RhoB\textsuperscript{−/−} mice showed increase in RhoA expression of similar magnitude to the wild type mice (Online Figure X).

**RhoB Is Required for Hypoxic HIF-1 Stabilization In Vitro and In Vivo**

HIF-1\(\alpha\) participates in hypoxia-induced pulmonary vascular remodeling.\textsuperscript{33,36} To study the effect of RhoB on HIF activation, we used the human osteosarcoma cells stably expressing a luciferase reporter construct under the control of a hypoxia
response element cell line, which has been optimized and validated in studies of HIF inhibitors and hypoxia-induced HIF activation.\(^3\)\(^7\) CARhoB significantly enhanced HIF-driven luciferase expression under hypoxic conditions (1.8-fold increase, \(P < 0.05\), comparison with hypoxic controls), whereas DNRhoB and manumycin attenuated hypoxia-induced HIF activation (1.6- and 1.5-fold reduction in HIF activity, respectively, comparison with hypoxic controls) (Figure 8A). DNRhoA alone or in combination with DN-RhoB did not have an effect (Figure 8A).

Stabilization of HIF-1\(\alpha\) by RhoB in HPAECs was confirmed by Western blotting and immunostaining (Figure 8B, Figure 5. RhoB regulates hypoxia-induced growth responses in human pulmonary artery endothelial cells (HPAECs) (A) and human pulmonary artery smooth muscle cells (HPASMCs) (B). The cells overexpressing Ad GFP (Ad control), Ad constitutively activated RhoB (AdCARhoB), Ad dominant-negative RhoB (AdDNRhoB), Ad dominant-negative RhoA (AdDNrhoA) (alone or in combination with AdDNRhoB) were incubated in normoxia or in hypoxia for 48 h, as indicated. Manumycin (5 \(\mu\)mol/L) was added at the start of hypoxic exposure. \(*P < 0.05, **P < 0.01\), comparisons with normoxic control; \#\(P < 0.05, \#\#P < 0.01\), comparisons with hypoxic control, \(n = 5\).

Figure 6. Effects of imatinib and RhoB on platelet-derived growth factor (PDGF)-stimulated (A, B) and hypoxia-induced (C, D) human pulmonary artery smooth muscle cells (HPASMC) proliferation and migration. [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reduction was measured in cells expressing mutant Rho GTPases and incubated with PDGF-BB (20 \(\mu\)g/L) or exposed to hypoxia for 48 hours. Cell migration was studied 18 hours following treatment with PDGF or hypoxia. Manumycin (5 \(\mu\)mol/L) or imatinib (5 \(\mu\)mol/L) were added to the cells at the start of experiment. \(*P < 0.05, **P < 0.01\), comparisons with normoxic/untreated control; \#\(P < 0.05, \#\#P < 0.01\), comparisons with hypoxic/PDGF-treated control, as appropriate. \(n = 5\).
HIF-1α expression and nuclear localization was reduced in cells overexpressing DNRhoB under hypoxic conditions but not in cells overexpressing DNRhoA (Figure 8B, 8E, 8F).

Chronic hypoxia significantly increased lung HIF-1α levels in wild type mice (1.5-fold increase, \( P \leq 0.05 \), compared with normoxic wild type controls) (Figure 8C and 8F). Consistent with the hypothesized role of RhoB, the lungs from chronically hypoxic RhoB-deficient mice showed reduced accumulation of HIF-1α (2.5-fold reduction, \( P \leq 0.05 \), comparison with hypoxic wild type controls) (Figure 8C and 8F). Changes in HIF accumulation were paralleled by changes in the expression levels of the PCNA. RhoB−/− mouse lungs showed significantly reduced PCNA expression (1.8-fold reduction, compared with hypoxic wild type controls, Figure 8D and 8F), consistent with decreased vascular remodeling.

Discussion

This study addresses for the first time the key integral role of RhoB in the regulation of the pulmonary vascular responses to hypoxia. We show that RhoB expression and activity are increased by hypoxia in cultured pulmonary vascular cells, coincident with the activation of RhoA. RhoB regulates cell proliferation, endothelial barrier function, and cell contractility and contributes to hypoxia-induced stabilization of HIF-1α. Chronic hypoxia significantly increases RhoB expression in the lung and RhoB gene knockout in mice attenuates chronic hypoxia-induced pulmonary hypertension, in spite of a compensatory increase in RhoA expression. Inhibition of RhoB farnesylation prevents the effects of hypoxia, an observation that might be exploited therapeutically using farnesyltransferase inhibitors.

We show for the first time that manipulation of RhoB activity affects endothelial barrier function. Activation of...
RhoA has previously been implicated in hypoxia-induced increase in pulmonary endothelial permeability. Activated RhoA induces stress fiber formation in endothelial cells as a result of increased actomyosin contractility. Increases in centripetal forces within cells counteract tethering forces created by intercellular adhesion molecules, compromising endothelial barrier function. Interestingly, we observed that inhibition of either RhoA or RhoB prevented hypoxia-induced increase in endothelial permeability in HPAECs, indicating that both GTPases were important, and consistent with their complementary effects on stress fiber formation.

Although there are reports of antineoplastic effects of RhoB in some types of cancer, it has growth-promoting effects in numerous cell types. Both RhoA and RhoB appeared to cooperate in hypoxia-induced cytoskeletal remodeling, but RhoB played a unique role in the regulation of pulmonary vascular growth responses to hypoxia. The effects of RhoB on cell growth under hypoxic conditions are likely to be mediated by multiple effectors. RhoB is required for the actions of several growth factors induced by hypoxia and regulates endocytotic trafficking of proproliferative and anti-apoptotic kinases Src and Akt. There is also evidence that RhoB can activate proinflammatory transcription factor NFkB to much greater extent than RhoA, a fact of potential importance in the regulation of inflammatory responses in the remodeled hypoxic lung. Here we show that the effect of RhoB on HPASMC proliferation is likely to be mediated by PDGF/PDGFR signaling. Imatinib, an inhibitor of PDGF receptor tyrosine kinase, abrogated the effects of overexpressing RhoB on HPASMC growth in vitro.

We also show that the effect of RhoB on hypoxia-induced pulmonary vascular responses in vitro and in vivo involve stabilization of HIF-1α, a transcription factor implicated in hypoxia-induced activation of PDGF receptor and pulmonary

Figure 8. RhoB is required for hypoxia inducible factor (HIF) stabilization in vitro and in vivo. (A) HIF activity in human osteosarcoma cells stably expressing a luciferase reporter construct under the control of a hypoxia response element (U2OS-HRE—luc) cells measured by luciferase reporter assay following 24 h exposure to hypoxia. (B) HIF-1α protein expression in human pulmonary artery endothelial cells (HPAECs); (C, D) HIF-1α and proliferating cell nuclear antigen (PCNA) protein expression in lungs from wild type (WT) and RhoB−/− mice, as indicated. (E) Inhibition of HIF-1α by dominant-negative RhoB (DNRhob) but not dominant-negative RhoA (DNrha) in HPAECs by immunofluorescence and confocal microscopy; Bar=10 μm. Representative examples of HIF-1α and PCNA protein expression, Western blotting. Cells and animals were treated as indicated; manumycin was added at the start of hypoxic exposure. Gray bars show normoxic HIF-1α levels, whereas black bars show hypoxic HIF-1α levels; n=4 (A, B) and n=4 (C, D); *P<0.05; **P<0.01, compared with hypoxic controls (A, B) or compared with hypoxic WT controls (C, D).
vascular remodeling. RhoB prevents proteolytic degradation of HIF-1α by the Akt/glycogen synthase kinase-3β pathway in glioblastoma cells. A relevant observation is that GTPase Rac1, which activates RhoB promoter, has been shown to stabilize HIF-1α in hypoxic Hep3B cells.

These observations translate in vivo. RhoB−/− mice exposed to chronic hypoxia developed mild pulmonary hypertension, with reduced vascular remodeling and right ventricular hypertrophy. This phenotype was associated with reduced HIF-1α and PCNA, a marker of cell proliferation, levels in the lung, akin to chronically hypoxic HIF-1α−/− knockout mice. Interestingly, a marked increase in RhoA expression was noted in the lungs of RhoB−/− mice. We did not seek to determine which cell types were responsible for the compensatory increase in RhoA expression. There is evidence this response is cell-/tissue-specific, as RhoA protein levels have been reported to be increased in macrophages but not in the hippocampus of RhoB−/− mice. RhoA expression was not affected by manipulating RhoB in cultured vascular cells in this study or in published reports. The data suggest that RhoB activity can be inhibited independently in vivo to reduce pulmonary artery pressure in pulmonary hypertension.

The contribution of RhoB to vessel contractility in vivo remains to be established. Our preliminary data show no significant differences in contractile responses to high K+, U46619, and phenylephrine or vasodilatory responses to sodium nitroprusside and acetylcholine in isolated intrapulmonary arteries from wild type and RhoB−/− mice (Online Figure XI).

RhoB is the only member of Rho GTPase family of proteins that is rapidly and transiently upregulated in response to stress conditions and our results show that gangliosylated RhoB has antioncogenic and proapoptotic effects, although some reports show no differential effects. The antioncogenic effects of farnesyltransferase inhibitors are thought to result from an increase in the levels of geranylgeranylated RhoB. In human pulmonary vascular cells, manumycin inhibited hypoxia-induced RhoB activation and prevented hypoxia-induced cell responses. Considering the rapid turnover of RhoB protein in cells, most of the effects of manumycin can be attributed to changes in RhoB activity. However, the proapoptotic effects of a more prolonged (>20 hours) treatment with manumycin may result from changes in farnesylation of other proteins important in the regulation of cell survival and metabolism such as Ras, Rheb, or nuclear lamins. Farnesyltransferase inhibitors can increase the levels of geranylgeranylated RhoB in some cell types resulting in increased apoptosis or stress fiber formation, but neither of these responses was observed in pulmonary vascular cells, suggesting that most of the active RhoB in pulmonary vascular cells might be farnesylated.

In conclusion, our work demonstrates the importance of RhoB as a mediator of hypoxia-induced pulmonary vascular remodeling and brings into focus its potential use as a drug target in pulmonary hypertension. As RhoB can compensate for the loss of function of RhoA, future research will need to establish whether targeting RhoB by farnesyltransferase inhibitors provide a therapeutic advance on less specific prenylation inhibitors, such as statins.

Acknowledgments
We thank Prof. Brian Morris (University of Glasgow, UK) for providing RhoB−/− mice and Dr Margaret Ashcroft (University College London, UK) and the Institute for Cancer Research, London, UK, for the use of U2OS cells.

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Disclosures
None.

References


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**Novelty and Significance**

**What Is Known?**

- Chronic exposure to hypoxia induces pulmonary hypertension (PH), which is characterized by pulmonary vascular endothelial dysfunction, vasoconstriction, and vascular smooth muscle hypertrophy.
- RhoA and Rho kinase are known to contribute to the vascular remodeling in PH but do not provide a complete explanation.
- Accumulating evidence suggests that RhoB, a protein similar to RhoA and important in cancer, may also regulate pulmonary vascular function.

**What New Information Does This Article Contribute?**

- RhoB is activated in pulmonary vascular cells exposed to hypoxia in culture and in the lungs of chronically hypoxic mice with PH.
- RhoB regulates endothelial barrier function, cell proliferation, and migration in hypoxic conditions by stabilizing hypoxia inducible factor 1α and enhancing cell responses to growth factors such as platelet derived growth factor.

- Genetic deletion of RhoB attenuates development of chronic hypoxia-induced PH in mice.

RhoB is a stress response protein abundant in the lung. We show that activation of RhoB plays a key role in regulating the pulmonary vascular response to hypoxia. Specifically, overexpression of RhoB increases pulmonary endothelial barrier permeability and pulmonary vascular cell migration and proliferation in culture. Inhibition of RhoB, using either a dominant-negative mutant or a farnesyltransferase inhibitor, inhibits the effects of hypoxia. RhoB knockout mice are protected from PH on exposure to hypoxia, as evident from reduced pulmonary vascular remodeling and right heart hypertrophy. The effects of RhoB are mediated, at least in part, by stabilization of transcription factor hypoxia inducible factor 1α. Inhibition of RhoB farnesylation might be exploited in future therapies aimed at regulating pulmonary vascular tone and structure.
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In the *Circulation Research* article by Wojciak-Stothard et al. (Wojciak-Stothard B, Zhao L, Oliver E, Dubois O, Wu Y, Kardassis D, Vasilaki E, Huang M, Mitchell JA, Harrington LS, Prendergast GC, Wilkins MR. Role of RhoB in the regulation of pulmonary endothelial and smooth muscle cell responses to hypoxia. *Circ Res*. 2012;110:1423–1434. DOI: 10.1161/CIRCRESAHA.112.264473) an author’s name was incorrect and should have been Louise S. Harrington.

The revised author list is as follows:

Beata Wojciak-Stothard, Lan Zhao, Eduardo Oliver, Olivier Dubois, Yixing Wu, Dimitris Kardassis, Eleftheria Vasilaki, Minzhou Huang, Jane A. Mitchell, Louise S. Harrington, George C. Prendergast, Martin R. Wilkins

This error has been corrected in the online version of the article, which is available at http://circres.ahajournals.org/content/110/11/1423.full.
Supplemental Materials and Methods.

Cell culture. Human pulmonary artery endothelial cells (HPAECs) were grown on fibronectin-covered (bovine fibronectin, Sigma-Aldrich Company Ltd. Gillingham, Dorset, UK) plastic ware in Human Endothelial Cell Growth Medium 2 supplemented with 2% foetal calf serum and EGF (5 µg/L), basic FGF (10 µg/L), IGF (20 µg/L), VEGF (0.5 µg/L), ascorbic acid (1mg/L), heparin (22.5 mg/L) and hydrocortisone (0.5 mg/L). Human pulmonary artery smooth muscle (HPASMCs) were grown in untreated plastic ware in the Human Smooth Muscle Cell Growth Medium 2 supplemented with 5% Foetal calf serum, EGF (0.5 µg/L) and FGF (2 µg/L). The cells were cultured under normal oxygen tension (20% O₂, 5% CO₂) or exposed to hypoxia (2% O₂, 5% CO₂, 92% N₂) for 1-48 hr. The cells and culture media were from PromoCell (Heidelberg, Germany). Prior to hypoxic exposure, the cells were treated with inhibitors or were infected with adenoviruses to express mutant RhoB proteins. Following the treatment, the cells were used for studies of Rho GTPases expression, activity, F-actin organisation, endothelial barrier function, MLC phosphorylation, cell proliferation, apoptosis and intracellular calcium measurement.

Semiquantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). To characterise changes in the mRNA expression profile of Rho isoforms in HPAECs and HPASMCs, total RNA was isolated using Trizol (Invitrogen) extraction and isopropanol precipitation. Prior to cDNA synthesis, the RNA was treated with DNase I (Invitrogen) to remove any residual genomic DNA. 1 µg of total RNA was used for cDNA synthesis. First-strand cDNA synthesis was performed using Superscript II reverse transcriptase (Invitrogen). RT–PCR was performed with isoform-specific primers:

- **RhoA**
  - Forward: 5’-CAGAAAAGTGACCCCAGAA
  - Reverse: 5’-GCAGCTCTCAGGTACCCATTTC

- **RhoB**
  - Forward: 5’-GAGAACATCCCCGAGAAGTG
  - Reverse: 5’-CTTCCTTGGTGCTTGGCAGAG

- **GAPDH**
  - Forward: 5’-CCTGGCCAAGGTCATCCATGACA
  - Reverse: 5’-GGGATGACCTTGCCCAC AGCCTT

For the analysis of RhoA and RhoB gene expression in mouse lung tissues, mouse primers and PCR conditions were used as in 1. 30 amplification cycles was chosen following the analysis of amplification profile and was confirmed to be within the exponential concentration range of the products (Online Figure XII).

Rho GTPases protein expression and activity. RhoA and RhoB protein expression in cells and tissues was studied by Western blotting. Active RhoA and RhoB were measured with recombinant GST-rhotekin Rho-binding domain (RBD) bound to glutathione beads (Amersham Biosciences), using 100 µg GST-RBD for each sample and detected by Western blotting as previously described 2.

Adenoviral infection. Adenoviral constructs for the dominant negative RhoB (DNRhoB; Ad-6myc-N19RhoB-GFP), constitutively activated RhoB (CARhoB; Ad-HA-V14RhoB-GFP) and dominant negative RhoA (DNRhoA; Ad-Flag-N19RhoA) as well as adenoviral control (Ad GFP) were made using Ad5 ΔE1ΔE3 backbone vector. Ad-HA-V14 RhoB-GFP was from (Welgen, Inc, Worcester, MA) while Ad-Flag-N19RhoA was from Cell Biolabs, Inc (San Diego, USA). Ad-6myc-N19RhoB was made as described in 3. Briefly, the N19RhoB cDNA was subcloned into the mammalian expression vector pcDNA3 in fusion with the 6myc epitope tag. The cDNA of RhoBN19 was cloned into KpnI and XbaI restriction sites of pAdTrackCMV vector. The recombinant adenoviruses were constructed using the Ad-Easy-1 system, where the adenovirus construct is generated in bacteria BJ-5183 cells. The recombinant adenoviruses were amplified in HEK293 cells and subsequently purified on 2 sequential cesium chloride gradients and then passed through PD10 columns (GE Healthcare) to reduce the salt concentration. The titer of the purified virus was estimated measured using A260 4. Briefly, the viral concentrate was diluted 1:20 in 0.1% SDS buffer and incubated at room temperature for 15 min, and then OD value was measured at A260. The concentration of adenovirus vector virions was determined by multiplying the absorbance by
the dilution factor (x 20) and then dividing by the extinction coefficient for wild-type adenovirus (ε_{260} = 9.09 \times 10^{-13} \text{ OD} \text{ ml cm virion}^{-1})^{d}. To establish numbers of active viral particles, adenoviral titre was measured in plaque assay. The titre of Ad-6myc-N19RhoB-GFP was 5.8 \times 10^{13} \text{ opu (optical particle unit)/ml} and 5 \times 10^{11} \text{ pfu (plaque forming units)/ml}; Ad-HA-V14RhoB-GFP was 1.04 \times 10^{13} \text{ opu/ml and 8 \times 10^{10} \text{ pfu/ml}}, \text{Ad-Flag-N19RhoA was 1.5 \times 10^{12} \text{ opu/ml and 1x10^{11} \text{ pfu/ml and Ad-GFP was 1.03 \times 10^{12} \text{ opu/ml and 1x10^{11} \text{ pfu/ml}}} \text{. Adenoviral transient infections of cells using multiplicity of infection MOI 1:100 lead to a maximal rate of infection efficiency (~90-95%) without obvious cytopathic effects. In experiments involving a short-term (1-2 hr) hypoxia, adenoviruses were added to the cells ~22 hr before the start of experiment (~24 hr over expression). In experiments involving 24-48 hr hypoxia, the adenoviruses were added to the cells 4 hr before the start of hypoxic exposure. Mutant protein expression was confirmed by immunofluorescence and Western blotting.}

Other treatments. In experiments involving a short-term (1-2 hr) exposure to hypoxia (permeability, calcium content, immunostaining of VE-cadherin and MLC phosphorylation), farnesyltransferase inhibitor, manumycin (5 \text{ µmol/L}, Enzo Life Sciences, ALX-350-241) was added to the cells 1 hr before the hypoxic exposure. In experiments involving a more prolonged exposure to hypoxia (6 – 48 hr), manumycin was added to the cells at the start of hypoxic exposure. Rho kinase inhibitor, Y-27632 (5 \text{ µmol/L}, Calbiochem) was added to the cells over expressing CARhoB for 6 hours, before cell fixation and immunostaining. The involvement of mA was verified by protein knockdown with mA siRNA (hDiaph1, Thermo Scientific Dharmacon), mA siRNA or non-targeting siRNA#2, Thermo Scientific, Dharmacon was introduced to cells by lipofectamine transfection and the experiments were carried out 72 hours post-transfection. mA siRNA was co-transfected with pcDNA3GFP to help visualise the transfected cells.

Myosin light chain (MLC) phosphorylation on Ser19 was studied by Western blotting. Mouse monoclonal anti-P-Ser19 MLC and rabbit monoclonal anti-MLC antibodies (Cell Signalling) were used at 1:200.

Endothelial permeability. Changes in endothelial barrier function were studied in Transwell filters (VWR) by measurement of passage of fluorescent dextran (FITC-dextran, MW 40 kDa, Sigma) through the endothelial cell layer.

Cell metabolic activity An MTS tertazolium colorimetric assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay; Promega) was used to assess metabolic activity associated with cell proliferation and migration, the amount of absorbance at 490 nm being proportional to the number of living cells in culture, irrespective of the presence of oxidative respiration. Proliferative responses of HPAECs and HPASMCs to hypoxia were studied in cells grown on 96-well plates (7 \times 10^4 \text{ cell/well}). The cells were left untreated or were incubated with adenoviruses for 4 hr. Following the incubation, fresh culture medium was added to the cells, containing manumycin (5 \text{ µmol/L}) or imatinib mesylate (Enzo Life sciences, 5 \text{ µmol/L}), as appropriate. The cells were then left in normoxia or were incubated in hypoxia for 48 hr. The effective concentration of imatinib was based on previously published results and its inhibitory effect on PDGF-β phosphorylation in HPASMCs was confirmed by Western blotting (Supplementary Figure 8).

In order to study the effects of RhoB on PDGF-induced HPASMC proliferation, adenoviruses were added to the cells for 4 hr before the start of hypoxic exposure in culture medium containing 1% serum and no growth factors. Fresh medium was then added to the cells containing different combinations of PDGF-BB (eBioscience, 20 µg/L), manumycin (5 \text{ µmol/L}) or imatinib (5 \text{ µmol/L}) and the cells were incubated in normoxic conditions for 48 hours.

Cell migration was measured in an in vitro wound assay. To study the effects of RhoB on PDGF-induced migration, confluent HPASMCs were treated with adenoviruses 4 hr before the addition of PDGF-BB and the inhibitors. Following 18 hr incubation, 1mm x 0.8 mm images of the wound edge were taken under the Olympus IX70 inverted fluorescent microscope using x 10 objective and F-view Soft Imaging System camera and the number of cells that that migrated out of the wound edge was
scored using Image Pro Plus image analysis software. 3 images/treatment were analysed in 3 separate experiments (n=9). The results were expressed as % of untreated controls. To analyse the effects of hypoxia on HPASMC migration, the cells were cultured in full medium and treated with adenoviruses or the inhibitors, as described above.

**HIF expression and activity.** HIF-1 stabilisation by RhoB was studied in a U2OS cell line stably expressing a luciferase reporter construct under the control of a hypoxia response element (U2OS-HRE-luc) (kind gift of Dr M. Ashcroft, UCL). U2OS-HRE-luc cell line had been optimised and validated in studies on HIF inhibitors and hypoxia-induced HIF activation. Changes in HIF-1α protein levels in HPAECs cultured in normoxic or hypoxic conditions for 6 hr were studied by Western blotting, while its localization was studied by immunofluorescence and confocal microscopy. Manumycin (5 µmol/L) was added at the start of hypoxic exposure.

**Apoptosis.** HPAECs or HPASMCs grown on optical (glass bottom) 96-well plates were left untreated or were over expressing GFP (adenoviral control) or RhoB mutant proteins or were treated with manumycin (5 µmol/L) for 48 hours. The cells treated with menadione (Sigma; 100 µmol/L, 6 hr) were used as a positive control. Tetramethylrhodamine, ethyl ester, perchlorate (TMRE) (40 nmoL/L; Invitrogen) was added to cells 30 minutes before the end of the experiment. The intensity of fluorescence, corresponding to the number of live mitochondria in cells, was analysed by confocal microscopy followed by image analysis (Adobe Photoshop CS5).

**Intracellular calcium levels** were studied with Rhod3 imaging kit (Molecular Probes), according to the manufacturer’s instructions. The cells were grown on 96-well optical bottom plates (Thermoscientific, cat no 165305). Rhod3 fluorescence, proportional to intracellular calcium content, was measured in a Glomax spectrophotometer (Promega) at excitation/emission 550/580 nm.

**Immunofluorescence and confocal microscopy.** The cells were cultured on plastic coverslips (Nunc, cat no 174950) and subjected to various treatments and then fixed with 4% formaldehyde solution in PBS for 10 minutes at room temperature and permeabilised for 3 minutes with 0.1% Triton X-100. Cells were incubated in 0.5% BSA in PBS for 45 minutes to block nonspecific antibody binding and then incubated with mouse anti-MLC2 (S19) antibody (Cell Signalling, 3675S), rabbit anti-MLC2 antibody (Cell Signalling, 3672S), mouse monoclonal anti-VE-cadherin antibody (Santa Cruz Biotechnology, sc-9989) or mouse monoclonal anti-HIF1-α antibody (ThermoScientific; MA1-16504) at 1:100 dilution. The coverslips were then washed 3 times in PBS and incubated with FITC-labelled rabbit anti-mouse antibody (Jackson Immunolabs, 115-095-068), Cy5-labelled goat anti-rabbit antibody (Jackson Immunolabs,111-175-144) or Cy5 goat anti-mouse antibody (Zymed, 81-6516) at 1:100 dilution and 1µg/ml TRITC-phalloidin (to stain actin filaments). Coverslips were washed 3x in PBS and mounted in Vectashield mounting containing nuclear stain DAPI. Images on cells were taken under the confocal laser scanning fluorescence microscopy (Leica TCS SP5).

**Construction of rhoB nullizygous mice.** The gene-targeting plasmid used to replace the single exon encoding the murine RhoB protein by homologous recombination was generated as follows. One-kilobase and 5.4-kb EcoRI genomic fragments from the murine rhoB locus were cloned into pBluescript SK(+) separately, generating the plasmids pR1 and pR5. pR1 was digested with ApaI and BamHI, blunt end filled, and ligated to a 4.8-kb XhoI-XbaI fragment containing an internal ribosome entry site (IRES)-tau-lacZ gene, generating pR120. pR5 was digested with BamHI and ligated to a 1.8-kb BamHI fragment containing pGKneo, generating pR51. The final targeting plasmid was generated by ligating a blunt-ended Sall-SpeI fragment from pR120 into the NotI site of pR51. Standard methods were used to electroporate Svi129 embryonic stem (ES) cells with a linearized preparation of the targeting construct produced by Sall digestion. Ten ES clones from 288 clones screened had undergone the desired homologous recombination event to replace one rhoB allele. Mouse C57B/6J blastocysts were injected by standard methods with three different targeted ES cell clones. Chimeric mice exhibiting germ line transfer of the...
targeted allele were obtained from all three clones. The genotype of mice and cultured embryo fibroblasts was confirmed by PCR and Southern blot analyses as described previously. Chronic hypoxia-induced mouse model of pulmonary hypertension. All studies were conducted in accordance with UK Home Office Animals (Scientific Procedures) Act 1986 and institutional guidelines. 12-15 weeks old C57BL male mice (20 g; Charles River, Margate, UK) and RhoB−/− male mice (a kind gift of Professor Brian Morris) were either housed in normal air or placed in a normobaric hypoxic chamber (FIO₂ 10%) for 2 weeks (n= 4-8/group). Development of PH was verified as described previously. At 2 week, animals were weighed and anesthetized (Hypnorm 0.25 mL/kg; Midazolam 25 mg/kg IP), and right ventricular systolic pressure (RVSP) was measured via direct cardiac puncture using a closed-chest technique in the spontaneously breathing, anesthetized animal. The animals were then euthanized, the hearts were removed, and the individual ventricular chambers were weighed, right ventricular hypertrophy was assessed as the ratio of right ventricle + septum with left ventricle + septum. The right lungs were snap-frozen in liquid nitrogen and stored at −80°C for biochemical measurements. The left lungs were fixed by inflation with 10% formalin, embedded in paraffin, and sectioned for histology. Transverse lung sections were stained with van Gieson’s elastic method or smooth muscle-actin antibody (Sigma). Vascular muscularisation was defined as the proportion of vessels (<50 µm diameter) with immunoreactivity for smooth muscle-actin (as evidence for muscularization) over the total number of vessels stained with elastin. Two separate sections from each animal were quantified, and counting was performed by two investigators blinded to genotypes.

Western blot analysis. Following protein electrophoresis and protein transfer, the membranes were probed with primary antibodies from the following sources: mouse monoclonal anti-RhoA (sc-418), rabbit anti-RhoB (sc-180), mouse monoclonal anti-VE-cadherin (sc-9989), mouse monoclonal anti-myoc (sc-40) and rabbit polyclonal anti-HA (sc-805) antibodies were from Santa Cruz Biotechnology; rabbit anti-Flag (F7425), mouse monoclonal anti-β-actin (A2228) were from Sigma; mouse monoclonal anti-HIF 1α antibody (MAI-16504) was from ThermoScientific and mouse monoclonal anti mDia antibody (610848) was from BD Biosciences Pharmingen. Secondary antibodies: goat anti-rabbit HRP-labelled (AG154) was from Sigma and goat anti-mouse HRP-labelled antibody (2016-07) was from Dako. Primary antibodies were used at dilution 1:1000 and secondary antibodies at dilution 1:5000. The relative intensity of the immunoreactive bands was determined by densitometry using Image J software (Rasband, W.S., ImageJ. U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2011). The results were normalised to β-actin levels and expressed as % of untreated controls. Untreated control samples were included in every experiment and all experiments were repeated at least 3 times (n≥3).

Measurement of PDGFR-β expression and phosphorylation. HPASMCs were grown in 6 cm Petri dishes until ~70% confluence and incubated overnight in medium deprived of growth factors and containing 1% serum. Imatinib (5 µg/L) was added to the cells 1hr before the treatment with PDGF PDGF-BB (20 µg/L) for 30 minutes. PDGFR-β was immunoprecipitated from the cell lysates following an o/n incubation of the lysates with the rabbit monoclonal anti-PDGFR-β antibody (Milipore, cat no. 05-825, 5 µg antibody/sample) with protein G sepharose beads (30 µl slurry/sample, 1 hr rotation at 4°C). The beads were washed 3x by centrifugation, boiled with the sample buffer, resolved by electrophoresis followed by Western blotting. The blots were subsequently probed with a rabbit monoclonal anti-PDGFR-β and a rabbit monoclonal anti-phospho-PDGFR-β antibodies (Milipore; 1: 1000).

Isometric wire myography. Vessel segments from RhoB male knockout mice or wildtype C57Bk6 mice were used. Mice were killed by pentobarbital injection and 2 intralobe pulmonary arteries were removed. Arteries were dissected free of fat and connective tissues, and the vessel segments placed in Mulvany wire myographs to measure contraction and relaxation responses. Throughout the experiment, tissues were immersed in physiological salt solution (PSS) 1.18 × 10⁻³ mol/L NaCl, 4.7 × 10⁻³ mol/L KCl, 1.17 × 10⁻³ mol/L MgSO₄, 2.5 × 10⁻³ mol/L CaCl₂, 1.0 × 10⁻³ mol/L KH₂PO₄, 2.7 × 10⁻⁵ mol/L EDTA, and 5.5 × 10⁻⁴ mol/L glucose, at 36 °C, bubbled with 95% O₂ and 5% CO₂. The tension of the vessel was normalised to a tension equivalent to that generated at 90% of the diameter of the vessel at
100 mmHg. Changes in arterial tone were recorded via a PowerLab/800 recording unit (ADI instruments Pty Ltd., Australia), and analysed using Chart 6.0 acquisition system (ADI instruments). Arteries were exposed to two challenges of high potassium solution (KPSS; 1.24 × 10^{-1} mol/L KCl, 1.17 × 10^{-3} mol/L MgSO₄, 2.5 × 10^{-3} mol/L CaCl₂, 1.0 × 10^{-3} mol/L KH₂PO₄, 2.7 × 10^{-5} mol/L EDTA, and 5.5 × 10^{-3} mol/L glucose) followed by a washout period of 10 min. Concentration response curves to 9,11-dideoxy-11α,9α-epoxymethanoprostaglandin F2α (U46619; 10^{-9}–10^{-7} mol/L) and phenylephrine (10^{-8} to 10^{-4} mol/L) were performed on each of the vessels. Dilatory response curves were recorded in pulmonary arteries pre-contracted with an EC80 concentration of U46619, and vasodilator responses to either acetylcholine (10^{-8}–10^{-4} mol/L) or SNP response curve (10^{-8}–3x10^{-5} mol/L) were assessed.

Statistical analysis. All the experiments were repeated at least 3 times (n≥3) and data are presented as the mean ± standard error of mean. Comparisons between groups were carried out using two-tailed Student t-tests or one-way ANOVA as appropriate. Statistical significance was accepted for P≤0.05 and tests were performed using GraphPad Prism version 4.0 or Microsoft Office Excel 2007.
Supplemental Figures and Figure Legends.

Online Figure I.

The effect of CARhoB (A, B) and Ad GFP (C) on cell morphology. HPAECs (A, C) and HPASMCs (B) were treated as indicated. The cells were fixed and stained for F-actin and VE-cadherin, as appropriate and studied under the confocal microscope. Bar=10 µm.
Online Figure II.

RhoB does not affect RhoA expression or activity. RhoA expression and activity were studied in HPAECs 24 hr after infection Adcontrol (AdGFP), Ad CARhoB (Ad-HA-V14RhoB-GFP) or Ad DNRhoB (Ad-6myc-N19RhoB-GFP). Representative Western blots are shown in (B). *P≤0.05, n=3.
Online Figure III.

Inhibition of hypoxia-induced stress fibre formation and dispersion of VE-cadherin in HPAECs over expressing Ad DNRhoA (Ad N19RhoA-flag, 24 hr over expression). The cells were fixed and stained for F-actin, VE-cadherin and flag epitope and studied under the confocal microscope. Western blot shows RhoA expression levels in untreated cells and cells over expressing AdDNRhoA-flag. Bar=10 µm.
Online Figure IV.

Manumycin inhibits RhoB activity without affecting RhoA activity in HPAECs. Graphs in (A,B) show changes in the activity of RhoB and RhoA in untreated HPAECs (control) or HPAECs cultured in hypoxic conditions for 2 hr, with or without manumycin (5 µmol/L), as indicated. (C) shows corresponding representative Western blots. Manumycin was added to the cells 1 hr before the start of hypoxic exposure. **P≤0.01, comparison with normoxic control; n=3.
Online Figure V.

RhoB mediates hypoxia-induced re-localization of MLC-P-Ser19 to stress fibres. HPAECs were left untreated or were infected with AdDNRhoB (Ad-HA-N19RhoB-GFP). 23 hr post-infection, the cells were left in normoxia or were exposed to hypoxia for 1 hr. Manumycin was added to the cells 1 hr before hypoxic exposure. The cells were then fixed and immunostained for F-actin (red), MLC-P-Ser19 (green) and GFP (blue), pseudocolors. Bar=10 µm.
Online Figure VI.

Inhibition of RhoA activity does not affect RhoB-induced stress fibre formation in HPAECs and HPASMCs. The cells were over expressing Ad CARhoB (Ad-HA-V14RhoB-GFP) together with Ad DNRhoA (Ad- N19RhoA-flag) for 24 hours. The cells were fixed and immunostained to visualise F-actin (red), GFP (green) and flag epitope (blue), confocal microscopy. Bar=10 µm.
Manipulation of RhoB activity in HPAECs and HPASMCs has no effect on intracellular calcium levels (Rhod3 fluorescence). The cells were incubated in normoxic or hypoxic conditions for 1 hr and were treated, as indicated. Manumycin was added to the cells 1 hr before the hypoxic exposure and ionomycin (0.5 µmol/L) was used as a positive control. *P≤0.05, **P≤0.01, comparisons with normoxic control, n=4.
Online Figure VIII.

Imatinib inhibits hypoxia- and RhoB-induced cell migration and RhoB activation in HPASMCs. (A) Selected images showing the effect of various treatments on HPASMC migration. The cells were treated, as indicated and cell migration was measured in wound assay 18 hr after wounding. Manumycin (5 µmol/L), imatinib (5 µmol/L) and PDGF-BB (20 µg/L) were added to the cells at the start of hypoxic exposure. Bar=10µm. (B) The inhibitory effect of imatinib on RhoB expression and activity in hypoxia (1 hr); *P≤0.05, **P≤0.01, compared with normoxic control, n=3. (C) Representative Western blots showing the effect of imatinib on PDGFR-β phosphorylation (PDGF-BB; 20 µg/L; 30 minutes) and hypoxia-induced increase in RhoB expression and activity (1 hr hypoxia). Imatinib (5 µmol/L) was added to the cells 1 hr before treatment with PDGF or hypoxia.
Online Figure IX.

Manipulation of RhoB activity in HPAECs and HPASMCs has no effect on cell apoptosis. The cells were incubated in normoxic or hypoxic conditions for 48 hr and were treated, as indicated. Manumycin (5 µg/L) was added at the start of hypoxic exposure and menadione (100 µmol/L, 6 hr) was used as a positive control. *P≤0.05; **P≤0.01, comparisons with untreated control. TMRE fluorescence; n=3.
Online Figure X.

RhoA and RhoB expression in the lungs of the wildtype (WT) and RhoB-/- mice. RhoB knockdown was confirmed by RT PCR and RhoA and RhoB protein expression in the lungs of normoxic (N) and hypoxic (H) mice were measured by Western blotting. **P≤0.01, comparisons with WT control; #P≤0.05, comparison between normoxic and hypoxic RhoB-/- groups; n=4-6.
Online Figure XI.

RhoB knockout mice show unchanged vasoconstrictor and vasodilator responses. Intrapulmonary vessels from RhoB−/− mice showed unchanged contractile responses to high [K+] (A), U46619 (B) and phenylephrine (C). Endothelial- independent vasodilation in response to sodium nitroprusside (SNP) was slightly attenuated in RhoB−/− mice (D), while endothelial- dependent vasodilator response to acetylcholine (E) was unchanged.
Online Figure XII.

Analysis of amplification profile of RhoA, semiquantitative RT PCR.

Supplemental References

