Inhibition of Rho-Kinase Attenuates Hypoxia-Induced Angiogenesis in the Pulmonary Circulation

Jean-Marc Hyvelin,* Katherine Howell,* Alistair Nichol, Christine M. Costello, Robert J. Preston, Paul McLoughlin

Abstract—Pulmonary hypertension (PH) is a common complication of chronic hypoxic lung diseases, which increase morbidity and mortality. Hypoxic PH has previously been attributed to structural changes in the pulmonary vasculature including narrowing of the vascular lumen and loss of vessels, which produce a fixed increase in resistance. Using quantitative stereology, we now show that chronic hypoxia caused PH and remodeling of the blood vessel walls in rats but that this remodeling did not lead to structural narrowing of the vascular lumen. Sustained inhibition of the RhoA/Rho-kinase pathway throughout the period of hypoxic exposure attenuated PH and prevented remodeling in intra-acinar vessels without enlarging the structurally determined lumen diameter. In chronically hypoxic lungs, acute Rho kinase inhibition markedly decreased PVR but did not alter the alveolar to arterial oxygen gap. In addition to increased vascular resistance, chronic hypoxia induced Rho kinase–dependent capillary angiogenesis. Thus, hypoxic PH was not caused by fixed structural changes in the vasculature but by sustained vasoconstriction, which was largely Rho kinase dependent. Importantly, this vasoconstriction had no role in ventilation-perfusion matching and optimization of gas exchange. Rho kinase also mediated hypoxia-induced capillary angiogenesis, a previously unrecognized but potentially important adaptive response.

Key Words: pulmonary hypertension ■ angiogenesis ■ RhoA ■ Rho-kinase ■ Y-27632

Sustained pulmonary hypertension (PH) is a common complication of chronic hypoxic lung diseases that is strongly associated with increased morbidity and reduced survival. Moreover, the presence of cor pulmonale is an independent predictor of increased mortality, suggesting that PH contributes directly to mortality (reviewed in Hopkins et al.). The increase in pulmonary vascular resistance (PVR) caused by chronic hypoxia has previously been attributed to structural changes in the vasculature, in particular encroachment of the remodeled arteriolar walls into the vascular lumen and loss of blood vessels, although recent reports have cast doubt on this paradigm.1–3 In particular, we have recently shown for the first time that hypoxia induces angiogenesis in the adult pulmonary circulation, a potentially beneficial adaptation, and does not cause vessel loss as previously believed.2 The small G-protein RhoA and its downstream effector Rho-kinase (ROCK) play a central role in diverse cellular functions including smooth muscle contraction, cytoskeletal rearrangement, cell migration, cell proliferation, and gene expression,4–8 Given these important functions, it is not surprising that disturbances of this pathway have been identified as important pathogenetic mechanisms in many diseases of the cardiovascular system, including systemic hypertension, arteriosclerosis, and ischemic heart disease.9–11 Blockade of the RhoA/ROCK pathway effectively corrects blood pressure in a number of animal models of systemic hypertension9,11 and is a key regulator of vascular smooth muscle proliferation and migration in disease-induced systemic vascular remodeling.9,10 This pathway also regulates endothelial cell proliferation and migration. In vitro models and in vivo Matrigel implants show that inhibition of RhoA or ROCK prevents growth factor–induced endothelial cell migration and organization into capillary-like structures12,13 and vessel formation.14 Moreover, ROCK inhibitors attenuate tumor growth in vivo in a manner that is compatible with an antiangiogenic effect.15 Recognition of the therapeutic potential of inhibiting this pathway has lead to the development of specific small molecule inhibitors of ROCK.8,11,15

Taken together, these reports suggest that the RhoA/ROCK pathway might contribute to the development of hypoxic PH and the associated pulmonary vascular remodeling. The purpose of the present study is to assess the potential role of this pathway in the development of chronic hypoxia-induced...
PH and the associated structural changes in the pulmonary vasculature. In particular, we wished to test the hypothesis that, although chronic inhibition of ROCK would inhibit the development of hypoxic PH, it would simultaneously inhibit hypoxia-induced pulmonary angiogenesis.

Materials and Methods
An expanded Materials and Methods section can be found in the online supplement available at http://circres.ahajournals.org.

Hypoxic Pulmonary Hypertension
Chronic hypoxic PH was induced by housing adult male rats in a normobaric hypoxic chamber (FiO₂ 0.10) for 1 or 3 weeks as previously described. Control rats were housed in normoxic conditions (FiO₂ 0.21) in the same room. To assess the effects of chronic ROCK inhibition, rats were randomized into 3 groups; 1 group was maintained in normoxic conditions (control group), while 2 groups were housed in hypoxic conditions for 1 week. Y-27632 was used to inhibit the phosphorylation activity of ROCK. One hypoxic group (CH-Y27632) received Y-27632 (30 mg·kg⁻¹·day⁻¹, orally) while the other did not receive Y-27632 (CH group).

Measurement of Vascular Pressures and Gas Exchange In Vivo
Rats were anesthetized, tracheostomised, paralysed, and mechanically ventilated. Venous and arterial cannulae were placed for drug administration, blood gas sampling, and blood pressure measurement. Right ventricular pressure was measured by introduction of a needle via a subdiaphragmatic approach just before euthanasia.

Isolated Perfused Lung Preparation
Lungs were isolated under anesthesia, mechanically ventilated, and perfused at constant low flow with a mixture of blood and physiological saline of constant hematocrit so that pulmonary arterial perfusion pressure (PAP) was a direct index of PVR. Capillary pressure was assessed by the double occlusion technique.

Tissue Collection
At the end of each isolated lung experiment, the left lung (LL) was fixed under standard conditions for stereological quantification of structural vascular changes. The right lungs were snap-frozen for subsequent protein and mRNA analysis.

Data Analysis
For stereological analyses, total volume of specific compartments, vessel and capillary lengths, and alveolar epithelial and capillary endothelial surface areas were reported per left lung. For normally distributed data, responses were reported as mean ± SEM, n refers to the number of rats from which tissue was obtained. For multiple comparisons of means across experimental groups, analysis of variance was performed followed by Student–Newman Keuls post-hoc test for pair-wise comparisons. For non-normally distributed data, multiple comparisons of medians across experimental groups were performed using the Kruskal–Wallis test followed by Mann–Whitney U with the Bonferroni post-hoc correction; values of P < 0.05 were accepted as statistically significant.

Results
Effect of Acute ROCK Inhibition on Established Hypoxic Pulmonary Hypertension
Mean peak right ventricular systolic pressure (RVSPₚₑₘₖ) measured during ventilation with 30% oxygen (53.5 ± 3.5 mm Hg) in rats (n=5) maintained chronically hypoxic for 3 weeks was significantly (P<0.01) greater than that in control nonhypoxic rats (28.8 ± 1.4 mm Hg), demonstrating the presence of fixed PH; ie, increased PVR that was not attributable to acute hypoxic pulmonary vasoconstriction (HPV). After administration of the potent ROCK inhibitor Y27632 (15 mg·kg⁻¹·day⁻¹), mean RVSPₚₑₘₖ was reduced significantly (P<0.01) to a value of 34.6 ± 5.6 mm Hg.

Changes in RVSPₚₑₘₖ in response to Y27632 in vivo may not have resulted from changes in PVR but may have resulted from changes in venous return, cardiac output, left atrial pressure, neurogenic control, and other factors. To examine the direct effect of ROCK inhibition on PVR in lungs chronically hypoxic for 3 weeks, we used isolated lung preparation. Mean PAP in chronically hypoxic lungs (n=5) was 15.1 ± 0.6 mm Hg and was significantly reduced (P<0.01) to 13.0 ± 0.9 mm Hg by Y27632 (3 μmol·l⁻¹). At the highest Y27632 concentration (100 μmol·l⁻¹) mean PAP was further reduced (P<0.01) to 10.7 ± 0.4 mm Hg, a value that was similar to that in control nonhypoxic lungs (see below and Figure 1).

Thus the dominant mechanism underlying chronic hypoxic PH was sustained vasoconstriction and not structural changes in the pulmonary vasculature. These results suggested that sustained ROCK inhibition in vivo would prevent the development of chronic hypoxic PH.

Chronic ROCK Inhibition In Vivo
To confirm that chronic exposure to a hypoxic environment for 1 week caused fixed PH, we measured RVSPₚₑₘₖ in anesthetized rats (n=5) during ventilation with 30% oxygen, and found that after such exposure RVSPₚₑₘₖ was significantly (P<0.01) elevated (42.5 ± 3.0 mm Hg) when compared with control nonhypoxic rats (28.8 ± 1.4 mm Hg). For the reasons outlined above, further investigation of the role of ROCK in the development of PH was undertaken using the isolated lung preparation.

Mean right ventricular (RV) weight (see supplemental Table I) in a group maintained hypoxic for 1 week (CH group) and in a hypoxic group chronically treated with Y-27632 (CH-Y27632) for 1 week were both significantly higher than that of the control group. The sustained treatment with ROCK inhibitor significantly reduced the RV hypertrophy compared with hypoxia alone although it did not alter the polycythemic response to hypoxia (supplemental Table I).

We assessed the total PVR in isolated lungs under 3 different conditions, ie, lungs ventilated with 16%, 7%, or 3%
O$_2$ (Figure 1). These concentrations give alveolar values that approximate normal sea level alveolar pO$_2$, the alveolar pO$_2$ experienced by the rats during hypoxic exposure in the chamber, and an alveolar pO$_2$ that produces a maximal hypoxic vasoconstrictor response. Lungs from the control group showed normal low PVR when ventilated with 16% O$_2$ and significant increases in resistance in response to reduction in alveolar O$_2$ indicating normal HPV (Figure 1). Chronic hypoxic lungs ventilated with 7% O$_2$ had significantly elevated vascular resistance compared with control lungs ventilated with the same inspired oxygen, and this significantly elevated vascular resistance compared with control lungs ventilated with the same inspired oxygen (Figure 1). Chronic inhibition of ROCK with Y-27632 had maximal effects on vascular resistance in the normal lung.

The A-aO$_2$ gap and PaO$_2$ were unchanged after injection of vehicle (data not shown).

**Effect of Chronic Inhibition of ROCK on Hypoxia-Induced Angiogenesis and Vascular Remodeling**

In a separate series of experiments, we assessed the effect of higher, potentially nonselective$^{8,11}$ concentrations of Y-27632 (up to 100 μmol/L) on the PAP in lungs maintained chronically hypoxic for 1 week and found that PAP was decreased to 10.1 (±0.2) mm Hg (n=3), a value similar to that in control lungs (9.6±0.3 mm Hg). These results were similar to those obtained in the 3-week hypoxic group and supported the conclusion that the chronic inhibition of ROCK, the structure of vessel walls was similar to that observed in the control group suggesting inhibition of hypoxia-induced vascular remodeling.

In the CH group, the mean total length of intra-acinar vessels (9236±630 cm/L$^{-1}$) was significantly higher than in the control group (5327±392 cm/L$^{-1}$, P<0.001). After chronic inhibition of ROCK, the total length of intra-acinar vessels (7864±508 cm/L$^{-1}$) was less than in the CH group although it remained significantly higher than in control group (P<0.01). Analysis of the mean lumen diameter of the intra-acinar vessels showed that there was no difference approximately two-thirds of the increase induced by chronic hypoxia through an effect that was mainly localized to the arterial (precapillary) segment (Figure 2B). This demonstrated that a vasoconstrictor activity of ROCK played a key role in maintaining chronic hypoxic PH.

Finally, in the CH-Y27632 group, in which the baseline PAP was similar to that in the control group, acute addition of Y-27632 had no significant effect on PA pressure in the isolated lungs, demonstrating that the chronic treatment with Y-27632 had maximally inhibited the effect of ROCK activity on PVR (Figure 2A).

In a separate series of experiments, we assessed the effect of higher, potentially nonselective$^{8,11}$ concentrations of Y-27632 (up to 100 μmol/L) on the PAP in lungs maintained chronically hypoxic for 1 week and found that PAP was decreased to 10.1 (±0.2) mm Hg (n=3), a value similar to that in control lungs (9.6±0.3 mm Hg). These results were similar to those obtained in the 3-week hypoxic group and supported the conclusion that the dominant mechanism underlying chronic hypoxic PH is sustained vasoconstriction.

In the presence of Y-27632 (3 μmol/L$^{-1}$), reducing the oxygen level to 3% did not produce significant pulmonary vasoconstriction in any group, indicating that ROCK is a key mediator of acute HPV (data not shown).

**Effect of Acute ROCK Inhibition on Pulmonary Oxygen Uptake in Hypoxic Rats**

In chronically hypoxic rats ventilated with a hypoxic inspirate (FiO$_2$ 0.10), there was no change in either the A-aO$_2$ gap or the partial pressure of oxygen in arterial blood (PaO$_2$) after acute administration of Y-27632 (see online supplement). The A-aO$_2$ gap and PaO$_2$ were unchanged after injection of vehicle (data not shown).
between the 3 groups (Figure 3A). However, the ratio of the wall thickness to the lumen diameter was significantly increased in the CH-group when compared with control and CH-Y27632 lungs (Figure 3B).

The total capillary volume and total capillary length (Table 1) were significantly increased in the CH group when compared with the control group, whereas after chronic treatment with Y-27632 these hypoxia-induced increases were significantly reduced and not significantly different from those in control group. The total capillary endothelial surface area was also significantly increased in the CH lungs when compared with the control group (Table 2). In the CH-Y-27632 group, capillary endothelial surface area was significantly less than that in the CH group although it remained elevated above the control value (Table 2). Chronic hypoxia also caused a significant increase in the total alveolar epithelial surface area when compared with the control group (Table 2), although in the CH-Y-27632 group this value was significantly less that in the CH group and similar to that in the control group. In both hypoxic groups, the increase in capillary endothelial surface area was proportionately greater than that in the epithelial surface area as indicated by the increase in the mean ratio of capillary endothelium to alveolar epithelium (Table 2).

**Effect of Hypoxia on RhoA, RhoA Activation, and ROCK Expression**

The total amount of RhoA was similar in each of the different groups of rats; ie, control, CH, and CH-Y-27632 (Figure 4). RhoA activation, expressed as the percentage of total RhoA in GTP-RhoA form, showed no significant difference between the different groups (Figure 4). In CH and CH-Y-27632 groups levels of ROCK I and II isoforms were significantly increased when compared with the control group (Figure 5).

**Effect of Chronic ROCK Inhibition on Vascular Growth Factor Gene Expression**

Chronic hypoxia increased expression of platelet-derived growth factor-α (PDGF-α), and this effect was significantly reduced by ROCK inhibition (Figure 6). Chronic ROCK inhibition also significantly reduced transforming growth factor β-1 (TGF-β1) expression when compared with chronically hypoxic lungs (Figure 6). Interestingly, expression of the proangiogenic genes vascular endothelial growth factor A (VEGF-A) and angiopoietin-1 (ANG-1) was not significantly altered at the end of one week of hypoxia and was unaffected by ROCK inhibition.

**Discussion**

It has been generally thought that PH predominantly resulted from hypoxia-induced structural changes in the pulmonary vasculature, which produced a fixed increase in resistance. These structural changes included remodeling of the arteriolar walls leading to encroachment into the vascular lumen, and loss of blood vessels, suggesting that interventions, which successfully ameliorated PH, would act by preventing or reversing such structural changes. The results of the present study cast doubt on this paradigm by demonstrating that chronic hypoxia did not cause a structurally based reduction of pulmonary vascular lumen diameter, nor did it cause a loss of pulmonary vessels. Moreover, we report that chronic inhibition of ROCK abrogated the development of hypoxic PH, not by preventing structural encroachment into the vascular lumen, but by inhibiting sustained pulmonary vasoconstriction. Importantly, we also found hypoxia-induced capillary angiogenesis in the adult lung that was dependent on the RhoA/ROCK pathway. ROCK inhibition reduced the expression of key vascular growth factors suggesting that this was one of the mechanisms by which it attenuated hypoxia-induced vascular remodeling.

Initially, we confirmed that exposure to hypoxia for three weeks induced fixed PH in vivo; ie, increased RVSP_{med} was not immediately reversed on exposure to normal alveolar oxygen and thus was not mediated by acute hypoxic pulmonary vasoconstriction. Previously, this observation, together with the extensive remodeling of pulmonary arterioles, has been taken as evidence that the increased PVR in chronic hypoxic PH was predominantly attributable to structural changes in the vasculature. However, in our experiments administration of the ROCK inhibitor Y-27632 produced a marked fall in PAP suggesting that ROCK-dependent vasoconstriction was the major determinant of hypoxic PH.

Because the changes in peak right ventricular systolic pressure in response to Y-27632 in vivo might not have been significantly increased when compared with the control group (Figure 5).

**TABLE 1.** Capillary Volume and Length

<table>
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<th>Control</th>
<th>CH</th>
<th>CH-Y-27632</th>
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<tbody>
<tr>
<td>Total capillary volume, cm³/LL⁻¹</td>
<td>0.158±0.004</td>
<td>0.217±0.015⁸</td>
<td>0.177±0.008</td>
</tr>
<tr>
<td>Total capillary length, ×10⁴ cm³/LL⁻¹</td>
<td>7.4±0.6</td>
<td>11.9±0.8⁶</td>
<td>8.6±0.6</td>
</tr>
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Values are mean±SEM. n=7 in all groups. *Significantly different from the 2 other groups (P<0.05).
attributable to changes in PVR but might have resulted from changes in other parameters, we used the isolated ventilated lung preparation to examine the direct effect of ROCK inhibition on PVR. Although the PA pressure in such preparations is much lower than in vivo values, it is a direct index of PVR. Our finding that \textit{Y}-27632 caused PAP to fall immediately to near normal values in lungs made chronically hypoxic for 3 weeks demonstrated that ROCK inhibition normalized PVR by a direct action on pulmonary resistance vessels and strongly suggested that hypoxic PH was not structurally based.

In view of the evidence that \textit{Y}-27632 could acutely reverse chronic hypoxic PH, we examined the effect of chronic \textit{Y}-27632 administration during 1 week of exposure to hypoxia. In agreement with previous reports, we first confirmed that exposure to hypoxia for 1 week produced marked PH in vivo, which persisted when alveolar oxygen was acutely elevated to supra-normal values (see Results), a finding that was confirmed in isolated lung preparations (Figure 1). In addition, we observed extensive vascular wall remodeling in CH lungs (Figure 3), as previously demonstrated after 1 week of hypoxia. Thus, these lungs demonstrated the features that had previously been interpreted as indicating structurally based PH. However, our stereological analysis demonstrated other hypoxia-induced structural changes in the pulmonary vasculature (angiogenesis and wall remodeling without lumen reduction) that differ radically from those suggested by the conventional paradigm (Figure 3). Why have such structural changes not been previously detected? The techniques we used to analyze vascular structure differ in 2 important ways from those previously used to address this problem, as we have previously discussed. First, stereology allows quantitative analysis of 3-dimensional structural parameters based on the 2-dimensional information available in histological sections and takes account of the changes in whole lung volume caused by chronic distending pressure that was constant at all points in the vasculature, was unaffected by changes in vascular resistance, and was identical in both control and hypertensive lungs. Thus the vascular dimensions measured in this study reflect structural change and were unaffected by pre-existing smooth muscle tone in vivo.

Our observation that chronic hypoxia did not cause encroachment of the remodeled vascular wall into the lumen is in good agreement with our previous report but extends those findings in 2 important ways. First, we show that this outward remodeling of the vessel wall occurred in lungs in which we had hemodynamically confirmed the development of fixed PH (Figure 1). Second, we found that chronic administration of \textit{Y}-27632, a selective ROCK blocker, inhibited the development of increased PVR in response to chronic hypoxia (Tables 1 and 2) and attenuated right ventricular hypertrophy in vivo (supplemental Table I), with-

### Table 2. Alveolar, Epithelial, and Capillary Endothelial Surface Areas and Ratio of Capillary to Alveolar Epithelial Surface Areas

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CH</th>
<th>CH-Y-27632</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total epithelial area, cm$^2$/cm$^3$</td>
<td>1909±149</td>
<td>2658±116*</td>
<td>2154±120</td>
</tr>
<tr>
<td>Total endothelial area, cm$^2$/cm$^3$</td>
<td>2250±160</td>
<td>4058±266*</td>
<td>3117±116*</td>
</tr>
<tr>
<td>Endothelial/epithelial ratio</td>
<td>1.19±0.03</td>
<td>1.52±0.05*</td>
<td>1.45±0.05*</td>
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Values are mean±SEM. n=7 in all groups. *Significantly different from the 2 other groups (P<0.05).

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**Figure 4.** A, Image of a sample Western blot of both GTP-RhoA and total RhoA in a single lung from each of the 3 different groups. B, Mean normalized densitometry of GTP-RhoA (GTP RhoA optical density in each lung expressed as a percentage of the total RhoA optical density in the lung), an index of RhoA activation. C, Mean total RhoA optical density in each of the 3 groups. N=7 in all groups; bars indicate SEM. Hela 60 cells used as positive control.

**Figure 5.** A, Sample Western blot of ROCK I and densitometric analysis showing mean optical densities in control, CH, and CH-Y-27632 lungs. B, Sample Western blot of ROCK II and densitometric analysis showing mean optical densities in each group. N=7 in all groups; bars indicate SEM. *significantly different from control (P<0.05).
out causing structural enlargement of the lumen (Figure 3). We confirmed that the dose used chronically in vivo was effective in blocking the contractile activity of the ROCK pathway by demonstrating that after isolation, these lungs showed no additional vasodilator response to acute Y-27632 administration (Figure 2). The failure of chronic ROCK inhibition to significantly enlarge mean-maximal-lumen diameter was observed, despite the fact that it successfully attenuated wall remodeling, a finding that is supported by the recent report of Fagan et al.21 Taken together, our results show that chronic hypoxic PH develops without structural change in the mean-maximal-vascular-lumen diameter and, furthermore, PH can be prevented without enlarging the structurally determined lumen diameter. This suggests that chronic hypoxia PH is not caused by structurally imposed changes in vascular resistance.

This conclusion is further reinforced by the demonstration that acute administration of a high concentration of Y-27632 normalized PVR in isolated lungs made chronically hypertensive by exposure to 1 and 3 weeks of hypoxia, both in vivo and in isolated lungs (Figure 2 and see Results), results that confirm the recent report of Nagaoka et al.22 Evidence supporting the important role of RhoA/ROCK is provided by the demonstration of a marked reduction in PVR (Figure 2) at concentrations of Y-27632 that are selective for ROCK (3 micromolar or less),8,11 although these concentrations did not return pulmonary arterial pressure to normal values. It has been previously shown that Y-27632 relaxes vascular smooth muscle, preconstricted by either agonists or hypoxia, by blocking ROCK-mediated phosphorylation of the regulatory protein CPI-17 and the MYPT-1 of myosin light chain phosphatase (MLCP), both of which effects activate MLCP, thus reducing myosin light chain phosphorylation and relaxing smooth muscle.15,21 Administration of higher concentrations of Y-27632 returned PVR to control values, although this vasodilator action might not have resulted from specific ROCK inhibition.5,8 Clearly, acute normalization could not occur if vascular resistance was increased by a fixed structural mechanism.

The RhoA/ROCK-dependent sustained vasoconstriction observed in chronically hypoxic lungs is distinct from that mediating acute HPV because acute normalization of alveolar oxygen did not reduce PVR after either 1 or 3 weeks of hypoxic exposure. This suggested that the sustained vasoconstriction of chronic hypoxia did not have a role in ventilation-perfusion matching. To test this directly, we examined the effect of acute ROCK inhibition on the alveolar-arterial oxygen gap, an index of physiological shunting, and found that this was unaltered (see online supplement). This suggests that chronic ROCK-mediated hypoxic PH is a maladaptive response, because it overloads the right ventricle without improving gas exchange and is in marked contrast to the beneficial optimization of ventilation-perfusion matching produced by acute HPV.

In the present study, we found that chronic hypoxia induced capillary angiogenesis in the adult lung in vivo (Tables 1 and 2), confirming the results of our previous report.2 In support of this Pascual et al reported that angiostatin, an inhibitor of angiogenesis, aggravated PH in the hypoxic lung, an observation that suggests new vessel formation in hypoxic lungs.23 More recently, Beppu and colleagues demonstrated hypoxia-induced capillary angiogenesis in the adult mouse lung.24 We now extend those findings by reporting that chronic ROCK inhibition significantly attenuated this hypoxia-induced angiogenesis (Tables 1 and 2). Inhibition of RhoA or ROCK has previously been shown to prevent growth factor–induced endothelial cell migration and organization into capillary-like structures in vitro and vessel formation in Matrigel implant.12,13,15,25,26 Our finding that Y-27632 attenuated hypoxia-induced angiogenesis in the lung is, to our knowledge, the first report directly demonstrating that the RhoA/ROCK pathway has a central role in hypoxic angiogenesis within adult tissues in vivo. In the lung, this may be particularly important as hypoxic angiogenesis increases the diffusing capacity for oxygen and may therefore be an important adaptive response to hypoxia.2 The possibility that hypoxia-induced angiogenesis might improve oxygen uptake in the lung awaits direct functional evidence. However, studies in high altitude and burrowing animals adapted to hypoxic environments show that the enlarged pulmonary capillary bed found in such species enhances oxygen uptake during exercise in hypoxia when oxygen uptake becomes diffusion limited, but not at rest when uptake is perfusion limited.25

The action of ROCK in the chronically hypoxic lung was not the result of increased expression of RhoA, a finding in good agreement with the recent report of Jernigan et al.28 Moreover, ROCK activity could not have resulted from increased RhoA activation because GTP-RhoA was reduced (although not significantly) and not increased (Figure 4). This result is similar to that of Baille et al who showed reduced RhoA activation in chronically hypoxic pulmonary vascular smooth muscle,29 although increased RhoA activation has been reported in chronically hypoxic lungs after 4 weeks.28 The ROCK effects that we observed may have been mediated through the increased expression of both its isoforms (Figure 5), a finding in good agreement with the demonstration that hypoxia augments expression of this enzyme in cultured pulmonary smooth muscle cells.30

ROCK activity is an important regulator of cytoskeletal rearrangement in endothelial cells during migration, and blockade of this action inhibits in vitro organization of endothelial cells into capillary structures.14,26 Such inhibition of cytoskeletal reorganization could have contributed to the attenuated angiogenesis that we observed in the CH-Y-27632 group. We explored the possibility that inhibition of remodeling and angiogenesis might also have been mediated by altered expression of key vascular growth factors. Our finding
that chronic Y-27632 treatment reduced PDGF-α and TGF-β1 expression (Figure 6) suggests that alteration of these growth factors might be an important mechanism by which ROCK inhibition attenuated arteriolar wall remodeling. Interestingly, neither chronic hypoxia nor ROCK inhibition changed the expression of VEGF-A or ANG-1 at 1 week (Figure 6). It is possible that expression of these genes might have been altered by hypoxia at earlier time points and that Y-27632 had blocked these changes, thus inhibiting angiogenesis. Alternatively other proangiogenic genes may play a predominant role in the lung.

It has recently been suggested that inhibitors of the RhoA/ROCK pathway might be useful therapeutic agents in the treatment of PH. In this context our findings are particularly noteworthy because they suggest that such inhibitors might act as a “double-edged sword”. Successful attenuation of PH would be expected to relieve right ventricular strain and the resultant adverse consequences. However, reduction in hypoxia-induced angiogenesis in the lung might simultaneously have an adverse effect on pulmonary diffusing capacity for oxygen, which might be a previously unsuspected adverse consequence of such a therapeutic approach. This possible effect of ROCK inhibition will require further investigation.

In summary, we found that increased expression of ROCK is a key signaling event in hypoxia-induced vascular adaptations in the lung. Activation of this protein kinase leads to chronic hypoxic PH, not by mediating structural reduction of the lumen of pulmonary blood vessels, but by causing sustained vasoconstriction. In contrast, the ROCK-dependent capillary angiogenesis that we report here may be a beneficial adaptive response, improving oxygen uptake in the lung particularly during exercise.

Acknowledgments

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References

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Expanded Materials and Methods

All study protocols were approved by the University Ethics Committee and conducted under license from the Department of Health.

Hypoxic pulmonary hypertension

Chronic hypoxic pulmonary hypertension was induced by housing adult male rats in a normobaric hypoxic chamber (FiO\(_2\) 0.10) for one or three weeks as previously described \(^1\). Control rats were housed in normoxic conditions (FiO\(_2\) 0.21) in the same room. To assess the effects of chronic ROCK inhibition, rats were randomized into three groups, one of which was maintained in normoxic conditions (control group) while two groups were housed in hypoxic conditions for one week. One hypoxic group (CH-Y27632) received Y-27632 (30mg.kg\(^{-1}\).day\(^{-1}\), orally) while the other did not receive Y-27632 (CH group). Y–27632 was purchased from Tocris (UK).

Measurement of vascular pressures and gas exchange in vivo.

After induction of anesthesia (60mg.kg\(^{-1}\) sodium pentobarbital intra-peritoneally), a tracheostomy was performed, the lung were mechanically ventilated (FiO\(_2\), 0.3; rate, 90 breaths.min\(^{-1}\); tidal volume, 4.5 ml.Kg\(^{-1}\); positive end-expiratory pressure, 2.5 cm H\(_2\)O; 15-minute recruitments with positive end-expiratory pressure of 15 cm H\(_2\)O for 20 breaths), and carotid arterial and dorsal penile vein camulas were inserted. Anesthesia and muscle relaxation were maintained with intravenous infusions of alphaxalone-alphadolone (Saffan) and panucorium, respectively. Depth of anesthesia
was assessed by monitoring the hemodynamic response to paw-clamp. Stable physiological conditions were obtained before entering the animal into the protocol, and animals were excluded when baseline inclusion criteria (i.e., normal oxygenation, acid-base status, hemodynamic status and temperature) were not met. After 30 minutes stabilization, animals were ventilated with hypoxic gas mixture (10% O₂, 90%N₂) for 15 minutes then Y-27632 (15mg.Kg⁻¹) or vehicle were injected intravenously and animal kept under hypoxic condition for a further 15 minutes. This was the maximum dose of Y-27632 that could be tolerated because of the reduction in systemic arterial pressure that it caused. Systemic mean arterial pressure, peak airway pressure, and rectal temperature were recorded throughout. Arterial blood gases and exhaust gases were determined every 15 minutes. Alveolar-arterial O₂ gap calculation were made using the complete alveolar equation. In some experiments, right ventricular pressure was measured at end expiration by introduction of a needle (23g) via a sub-diaphragmatic approach just prior to euthanasia.

*Lung isolation and perfusion.*

An isolated ventilated perfused lung preparation was used to assess the effect of experimental interventions on total pulmonary vascular resistance, as previously described. In brief, rats were anaesthetized (60mg.kg⁻¹ sodium pentobarbital intraperitoneally), and mechanically ventilated (SAR-830P small-animal ventilator, CWE, Ardmore, PA) at a tidal volume of 1.8 ml and a respiratory frequency of 80 breaths.min⁻¹ as previously described. The animals were then anti-coagulated (1000 IU.Kg⁻¹ heparin intra-venously) and killed by exsanguination. The thoracic contents were exposed through a midline sternotomy, and cannulas were inserted into the main pulmonary artery and left atrium and tied in place. The thoracic contents
were removed en bloc and suspended in a chamber maintained at 37°C, while ventilation continued with a warmed and humidified gas mixture of 5% CO₂, 21% O₂, balance N₂ for the normoxic rats and 5% CO₂, 7% O₂, balance N₂ for the hypoxic rats. Airway pressure was continuously monitored, and a positive end-expiratory pressure of 2.0 cmH₂O was maintained. The lungs were briefly hyperinflated to an airway pressure of 16 cmH₂O every 5 minutes to prevent development of progressive atelectasis.

Lungs were perfused with a mixture of blood and physiological saline solution. The blood was collected from normoxic rats and mixed with PSS (in mmol/L: 121 NaCl, 21 NaHCO₃, 5.4 KCl, 4 MgSO₄, 1 NaH₂PO₄, 1.8 CaCl₂, 5.6 glucose plus 4% Ficoll) in a ration of 2:1. Perfusion was maintained at a constant flow (0.04 ml.min⁻¹.Kg⁻¹) so that changes in arterial perfusion pressure reflected changes in total pulmonary vascular resistance. Venous outflow pressure was maintained constant at 3.5 mmHg to ensure zone 3 conditions at the end of expiration. All measurements of arterial perfusion pressure were made at end of expiration. Arterial, venous and airway pressure were continuously recorded with an analog-to-digital system (Biopac MP100 WS, Linton Instrumentation, Norfolk, UK).

The baseline capillary pressure (Pc) was assessed by the double occlusion technique. Briefly inflow and venous outflow lines were simultaneously occluded and vascular pressure rapidly equilibrated to a new pressure representative of Pc. This allowed pre-capillary and post-capillary resistances to be calculated separately.
**ROCK inhibition**

Y–27632 was used to inhibit ROCK phosphorylation activity both in vivo and in isolated lung experiments. At concentrations less than 10 µm/L, this agent is a relatively selective ROCK inhibitor. Higher concentrations approach or exceed its Ki for other kinases that regulate smooth muscle tone including protein kinase A, protein kinase C especially novel isoforms, citron kinase and protein kinase N. We used high concentrations to determine whether pulmonary hypertension was structurally based or due to vascular smooth muscle contraction. However, it is important to note that the actions of this compound may then be due to effects additional to ROCK inhibition.

**Tissue collection**

At the end of each isolated lung experiment, the lung was ventilated with a gas mixture 5%CO₂, 16%O₂, balance N₂, and the pulmonary artery was perfused with normal saline (37°C) until the effluent was clear of blood. Calcium-free physiological saline solution was introduced into the pulmonary circulation to induce complete relaxation of the pulmonary vessels. A ligature was subsequently tied around the right main bronchus and the right branch of the pulmonary artery and the right lobes were removed and quickly snap frozen for further analysis. The left lung was fully inflated at a pressure of 25 cmH₂O with fixative (4% wt.vol⁻¹ paraformaldehyde), followed by simultaneous infusion of this solution through the pulmonary artery (62.5 cmH₂O). The cannula in the left atrium was clamped to obstruct the outflow from the pulmonary veins, so that the pulmonary vasculature was fixed under ‘no flow’ conditions. This maneuver produced a constant capillary transmural distending
pressure (37.5 cmH₂O) at all locations along the vascular bed. After one hour, the main-stem bronchi and pulmonary artery were tied off at the level of the hilum, and the left lung volume determined by water displacement, as previously described.

**Measurement of Right Ventricular weights**

The right ventricular free wall (RV) was dissected from the left ventricle and septum (LV + S), each ventricle weighed separately and the ratio of RV to LV + S weight calculated. Right ventricular weight was expressed in grams per 100 grams of body weight.

**Assessment of RhoA activation and ROCK level.**

Frozen lung tissue was homogenized as previously described. Briefly, lung tissue was homogenized using a polytron in 5 volumes of ice-cold homogenization buffer comprising (in mmol/L) 50 Tris-HCl (pH 7.2), 500 NaCl, 10 MgCl₂, 1 ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate (SDS), 20 µg.ml⁻¹ each leupeptinin and aprotinin and 1 mM phenylmethyl sulfonyl fluoride (PMSF). Homogenates were clarified by centrifugation at 5000g at 4°C for 10 min. Protein concentration was determined using a commercial protein assay kit (BCA Protein Assay, Pierce Biotechnology, Rokford, USA). A small amount of supernatant (7.5 µg of protein) was taken to determine the amount of total RhoA, ROCK-I and ROCK-II by western blot analysis. Equal amounts of protein (500 µg) were used to determine the amount of GTP-bound RhoA using Rhotekin immobilized onto glutathione-agarose beads (Upstate, UK). Each sample was then analyzed by SDS-15% polyacrylamide gels. Resolved proteins
were transferred onto nitrocellulose. Primary monoclonal antibody 26C4 (SantaCruz Biotechnology Inc.) was used at 1:6000 dilution for detection of RhoA, primary monoclonal antibodies anti ROCK-α or anti ROCK-β (BD Biosciences, UK) were used at 1:1000. Horseradish peroxidase-conjugated secondary goat antibody anti-mouse IgG (SantaCruz Biotechnology Inc.) was used at 1:8000. Membranes were incubated for 1h each with primary and secondary antibody diluted in TBS-Tween with 5% non-fat dry milk. The immunoreactive bands were detected by ECL (Amersham Biosciences, UK) and quantified by densitometric analysis (NIH Image 1.63, National Institutes of Health, USA). The extent of RhoA activation (GTP-RhoA) was expressed as the ratio of the density of the GTP-RhoA band to that of the total RhoA in each sample.

**Stereological assessment of vascular structural changes**

We examined the structural changes induced by hypoxia in arterial, capillary and venous vessels as previously described using isotropic uniform random sections obtained from each left lung. Briefly, the left lung was systematically cut into 4 mm thick slices starting from a randomly chosen position within the first 4 mm. The slices were embedded in resin and cut into bars at 2 mm intervals, and every third bar selected from a randomly chosen start point between one and three. Sections (1-2 μm) were taken from a random position within each tissue block and stained with Toluidine Blue. Random fields of view from each section were examined by light microscopy, captured using video camera (JVC KY-F55B Eurotec, Ireland) and imported into Stereology Toolbox (Morphometrix, USA) for analysis.

Volume densities of specific tissues in the lung (volume per unit volume of lung) were estimated by point counting, and the absolute volumes were calculated
using the previously measured lung volumes. The volume densities of the extra-acinar tissues (defined as large vessels and airways, down to and including terminal bronchioles, and their associated connective tissue, vasa vasorum and nerves) and the volume of intra-acinar tissues (defined as respiratory bronchioles, alveolar tissue and the associated vessels) were estimated by point counting. The intra-acinar gas exchange region of the lung was next considered as three sub-compartments: the intra-acinar blood vessels (that is all blood vessels excluding capillaries), the intra-acinar airspaces and, finally, the alveolar walls including capillaries. To estimate the volume densities of the vessel lumen and the vessel wall including tunica intima and tunica media, images of randomly selected intra-acinar vessels were captured and placed randomly within point counting grids. Capillary lumen volume density was estimated by point counting on random fields of view obtained at high magnification.

To estimate the length of intra-acinar vessels per unit lung volume (length density) the number of intra-acinar vessels that transected counting frames randomly superimposed over images taken from IUR sections was counted as previously described. The vessels were considered to be cylindrical in shape, thus the average radius of the intra-acinar vessels was calculated from the measured length and volume densities using standard formulae. Capillary length and volume density were calculated in the same manner as intra-acinar vessels.

*Extraction of RNA and real-time PCR (TaqMan) quantification*

Total RNA was extracted from whole lung samples, from control (n=4), CH (n=6) and CH-Y27632 (n=7) animals using RNeasy Mini columns (Qiagen; Germany) and reverse transcribed (RT) to cDNA using Superscript II RNase H-Reverse
Transcriptase kit (Invitrogen, USA). Probe and primer sequences were designed to non-redundant sequences (transforming growth factor, beta 1 (Tgfb1):

F-primer: 5’-GTCCCAAACGTCGAGGTGA-3’;

R-primer: 5’-CCATGAGGAGCAGGAAGGG-3’;

probe: 5’-TGGGCACCATCCATGACATGC-3’),

or ordered from ABI as Assays-on-Demand Gene Expression Assays: (i) platelet derived growth factor, alpha (Pdgfa; Assay ID: Rn00709363_m1), (ii) vascular endothelial growth factor A (Vegfa; Assay ID: Rn00582935_m1), and (iii) angiopoietin 1 (Angpt1; Assay ID: Rn00585552_m1). Probes were labeled with FAM. The Eukaryotic 18S rRNA (VIC) pre-developed assay reagent kit was used as the endogenous control. Reactions were carried out on the ABI PRISM 7900 Sequence Detection System, according to the TaqMan PCR protocol, and mRNA transcript levels were determined using the comparative Ct method (ABI Prism 7700 Sequence Detection System User Bulletin #2).

**Chemicals**

All salts and drugs were supplied by Sigma-Aldrich except where otherwise indicated.

**Data Analysis**

For stereological analyses, total volume of specific compartments, vessel and capillary lengths and alveolar epithelial and capillary endothelial surface areas were reported per left lung. For normally distributed data, responses were reported as...
means ± standard error of the mean (SEM), n refers to the number of rats from which tissue was obtained. For multiple comparisons of means across experimental groups, analysis of variance was carried out followed by Student-Newman Keuls *post hoc* test for pair-wise comparisons. For non-normally distributed data, multiple comparisons of medians across experimental groups were carried out using the Kruskal-Wallis test followed by Mann-Whitney-U with the Bonferonni *post hoc* correction; values of $P<0.05$ were accepted as statistically significant.
**Online Supplement Results**

*Effects of one week hypoxic exposure on body weight, right ventricle and hematocrit*

Mean body weights (Online Table 1) in a group maintained hypoxic for one week (CH group) and in a hypoxic group chronically treated with Y-27632, (CH-Y27632), were significantly lower than that of the control group, a well-established effect of chronic hypoxia. Chronic hypoxia also caused a significant increase in hematocrit (Online Table 1). In both hypoxic groups, right ventricular hypertrophy was evident, as demonstrated by the increased mean weight of the right ventricle (RV) and the increased ratio of right to left ventricular weight compared to the control group (Online Table 1). ROCK inhibitor treatment significantly reduced the hypoxia-induced RV hypertrophy although it did not alter the polycythemic response to hypoxia (Online Table 1).

*Effect of acute ROCK inhibition on pulmonary oxygen uptake in hypoxic rats.*

In chronically hypoxic rats ventilated with a hypoxic inspirate (FiO₂ 0.10) there was no change in either the A-aO₂ gap or the partial pressure of oxygen in arterial blood (PaO₂) following acute administration of Y-27632 (Online Figure 1). These results suggest that ROCK-dependent vasoconstriction in chronically hypoxic hypertensive lungs does not contribute to optimizing ventilation perfusion matching. The A-aO₂ gap and PaO₂ were unchanged after injection of vehicle (data not shown).

*Effect of chronic inhibition of ROCK on hypoxia-induced angiogenesis and vascular remodeling.* In the control lungs, the majority of intra-acinar vessels were thin-walled, with a single elastic lamina and no discernable tunica media (Online Figure 2A), findings typical of normal lungs with normal low pulmonary vascular resistance. In
the CH group remodeling of the wall of intra-acinar vessels was evident, as shown by
the development of separate internal and external elastic laminae and an intervening
layer of media (Online Figure 2B), a finding that is characteristic of the vascular
remodeling of chronic hypoxic pulmonary hypertension. Following chronic inhibition
of ROCK, the structure of vessel walls was similar to that observed in the control
group suggesting inhibition of hypoxia-induced vascular remodeling.
References for online supplement


8. Howell K, Preston RJ, McLoughlin P. Chronic hypoxia causes angiogenesis in addition to remodelling in the adult rat pulmonary circulation. In: 

Online Figure 1. Alveolar-arterial oxygen gap (A-aO₂ gap) and partial pressure of arterial oxygen (PaO₂) in CH rats in vivo ventilated with 10% oxygen, before (control) and after injection of Y-27632 (15mk/Kg iv).

Online Figure 2. Photomicrographs of intra-acinar blood vessels taken from control and CH rats. A, Thin walled, non-muscularised, intra-acinar vessel from a control lung showing a single elastic lamina (EL) surrounding the vessel lumen (VL). B, Completely muscularised, remodeled intra-acinar vessel from a CH lung showing separate internal (IEL) and external (EEL) elastic laminae and a medial layer (ML). Scale bars represent 50 µm.
Online Table 1. Body weights, hematocrit and ventricular weights.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CH</th>
<th>CH-Y-27632</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g)</td>
<td>357 ±10</td>
<td>310 ±12*</td>
<td>306 ±6*</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>0.50 ±0.10</td>
<td>0.71 ±0.10*</td>
<td>0.69 ±0.20*</td>
</tr>
<tr>
<td>RV weight (g.(100g.body wt)⁻¹)</td>
<td>0.08 ±0.02</td>
<td>0.13 ±0.01#</td>
<td>0.10 ±0.01#</td>
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<tr>
<td>RV/ (LV+S) ratio</td>
<td>0.39 ±0.01</td>
<td>0.61 ±0.02#</td>
<td>0.50 ±0.01#</td>
</tr>
</tbody>
</table>

Values are means (±SEM) * significantly different from control (P<0.05); n = 7 in all groups.

# significantly different from the two others groups (P<0.05).