Sildenafil Prevents Change in RhoA Expression Induced by Chronic Hypoxia in Rat Pulmonary Artery

Vincent Sauzeau, Malvyne Rolli-Derkindener, Stéphanie Lehoux, Gervaise Loirand, Pierre Pacaud

Abstract—Exposure to chronic hypoxia (CH) induces a sustained pulmonary hypertension associated with structural and functional changes in the pulmonary arterial bed, including alterations of contractile properties. The small G-protein RhoA and its effector Rho kinase play a major role in the sustained rise in tension induced by vasoconstrictors. The aim of this study was to analyze the effect of CH on the RhoA/Rho kinase signaling pathway in the rat pulmonary artery. Maximal contraction of pulmonary artery rings to endothelin-1, noradrenaline, and the thromboxane A2 analog U46619 was markedly decreased in rats exposed to CH (10% O2, 2 weeks). This CH-induced decrease response to agonists was attributable to the abolition of RhoA-mediated Ca2+ sensitization of the contraction. Real-time reverse transcriptase–polymerase chain reaction and Western blot analysis revealed a decrease in RhoA mRNA (79.4 ± 6.0%, n = 4) and RhoA (81.1 ± 8.0%, n = 4) expression in the main pulmonary artery from CH rats, whereas RhoA expression was not modified in arterial smooth muscle cells and arteries exposed to hypoxia and high intraluminal pressure, respectively. Treatment of rats with sildenafil (25 mg/kg per day) throughout 2 weeks of exposure to CH prevented CH-induced downregulation of RhoA, reduction of contraction, and pulmonary artery remodeling. These findings indicate that CH-induced downregulation of RhoA expression, leading to the abolition of RhoA/Rho kinase–mediated Ca2+ sensitization of contraction, is responsible for the decreased responses to contracting agonists in the pulmonary artery of CH rats. These alterations are prevented by sildenafil, indicating a major role of the NO/cyclic GMP pathway in CH-induced altered RhoA signaling in the pulmonary artery. (Circ Res. 2003;93:630-637.)

Key Words: smooth muscle • contraction • G proteins • pulmonary hypertension

Pulmonary hypertension (PHT) is characterized by high pulmonary blood pressure, vascular remodeling, and right ventricular hypertrophy. Whereas primary PHT results from unknown causes and genetic predisposition, secondary PHT is a common complication of pulmonary obstructive disease, lung diseases, and heart failure. The pathogenesis of secondary pulmonary hypertension is not fully understood, but hypoxia is considered a major factor. In many mammalian species, including humans, acute hypoxia causes a selective pulmonary arteriolar vasoconstriction and increases pulmonary blood pressure. Exposure to chronic hypoxia (CH) induces a sustained PHT associated with marked structural and functional changes in the pulmonary arterial bed.1,2 The remodeling of the pulmonary arterial wall includes smooth muscle cell proliferation and abnormal extracellular matrix protein deposition that decrease the lumen of the vessels and reduce the elasticity of the arterial wall.3,4 These structural changes also contribute to functional alterations, notably to modifications of contractile properties.

Besides extensive studies focused on the mechanisms involved in the pulmonary vasoconstrictor response to acute hypoxia,5,6 there is still no consensus regarding the alterations of contractile properties of pulmonary artery induced by CH and the mechanisms involved. Although impairment of the endothelium-dependent regulation of pulmonary vascular tone is consistently reported, the analysis of the role of nitric oxide (NO) and cyclic GMP (cGMP) signaling pathway in CH-induced PHT has yielded conflicting data, with both increase and decrease of endothelial NO synthase having been described.7–11 Regarding the reactivity of pulmonary artery smooth muscle to vasoconstrictors, inconsistent data have been reported depending on the agonists used, the duration of exposure to CH, the portion of the pulmonary vascular bed examined, and the animal species used. It has been described recently that the maximal contraction to endothelin (ET)-1 and angiotensin II was decreased in the main pulmonary artery from CH rats, whereas 5-hydroxytryptamine–mediated contraction was enhanced both in the first branch and small muscular pulmonary arteries.12,13 Such agonists that bind to G-protein–coupled receptors produced contraction by increasing both the cytosolic Ca2+ concentration and the Ca2+ sensitivity of the contractile apparatus.14 The contribution of altered Ca2+ signaling to CH-induced change in pulmonary arterial reactivity has been investigated.12,15 but the involvement of CH-induced alteration in Ca2+ sensitization has not been analyzed.
Ca⁡²⁺ sensitization mediated by the small G-protein RhoA and its target Rho kinase constitutes the major component of the sustained rise in tension induced by vasoconstrictors and contributes to arterial blood pressure regulation.¹⁶–¹⁸ Data are now accumulating regarding the involvement of Rho proteins and Rho kinase in arterial disorders associated with arterial wall remodeling, altered cell contractility, and cell migration, such as hypertension, atherosclerosis, and restenosis.¹⁶,¹⁹–²² Although a better knowledge of the effect of CH on the signaling mechanisms involved in the regulation of the contractile properties would help in understanding the pathophysiology of the pulmonary circulation, there is no data regarding the RhoA/Rho kinase pathway in this context.

The present study was thus designed to analyze the effect of CH on agonist-induced contraction and on RhoA/Rho kinase signaling in the rat pulmonary artery. We show that CH induced a downregulation of RhoA expression and RhoA/Rho kinase–mediated Ca²⁺ sensitization of the contraction, responsible for a decreased response to contracting agonists. These alterations are completely prevented by the oral administration of sildenafil to rats exposed to CH, indicating a major role of the NO/cGMP pathway in the CH-induced alteration of RhoA signaling in the pulmonary artery.

Materials and Methods

Animals

The normoxic rats were housed in room air at a normal atmospheric pressure (760 mm Hg). The hypoxic rats were housed in a hypobaric chamber (VacuCell 111 L, Medcenter) for 15 days. At completion of the exposure, tissues were prepared as indicated for contraction measurements, Western blot analysis, RNA extraction, or morphological analysis.

NO₃⁻ Assay

Left ventricular plasma NO₃⁻ concentration was determined by spectrophotometric analysis, as described previously.²³

Tension Measurements in Intact Fibers

The aorta and extralobar pulmonary artery rings were suspended under isometric conditions and connected to a force transducer (Pioden Controls Ltd). Cumulative concentration-response curves were constructed in response to KCl, endothelin (ET)-1, noradrenaline (NA), and the thromboxane A₂ receptor agonist U46619. Amplitude of the contraction was expressed as a percentage of the maximal KCl-induced contraction. The amplitude of relaxation was expressed as percentage of the maximal amplitude of contraction induced by phenylephrine recorded before carbachol (CCH) or sodium nitroprusside (SNP) application.

Isometric Tension Measurement in Skinned Fibers

Small muscle strips isolated from the media of extralobar pulmonary arteries were connected to a force transducer (AE 801, SensoNor) and permeabilized with β-escin (50 to 70 μmol/L) for 35 minutes at 25°C, as previously described.²⁴ Tension developed by permeabilized muscle strips was measured in activating solutions, containing 10 mmol/L EGTA and a specified amount of CaCl₂ to give a desired concentration of free Ca²⁺.²⁴

Western Blot Analysis

Expression of RhoA and Rho kinase was analyzed using mouse monoclonal anti-RhoA antibody or with goat polyclonal anti-Rho kinase I antibody, respectively. Immunoreactive bands were visualized using horseradish peroxidase–conjugated secondary antibodies and subsequent ECL detection (Amersham Pharmacia).

Real-Time RT-PCR

Total RNA was extracted using TRizol Reagent (Life Technologies), and reverse transcription was performed according to standard techniques. Quantitative real-time polymerase chain reaction (PCR) assays were carried out with sequence-specific primers pairs on the iCycler iQ system (BioRad) using intercalation of Sybr Green as fluorescent probe. The Sybr Green Kit (Perkin Elmer Applied Biosystems) was used for real-time monitoring of amplification. Results were evaluated by iCycler iQ Real Time Detection System software (BioRad). The expression of GAPDH mRNA was used to normalize the expression of RhoA mRNA.

Pressurized Arteries

Removed rat carotid artery segments were maintained in the organ culture system for 1 or 3 days, pressurized at normal (80 mm Hg) or hypertensive (150 mm Hg) levels. The device used for application of intraluminal pressure to vessel segments in organ culture has been described previously.²⁵

Morphological Analysis

Pulmonary arteries and aortas from control and hypoxic rats were collected and fixed for 1 hour with paraformaldehyde (4% wt/vol). Transverse sections (6 μm thick) were stained with Sirius red (Sigma) for collagen fibers and eosin-hematoxylin (Sigma) for nuclei and then analyzed using Metamorph software (Universal Imaging Co).

Chemicals and Drugs

Mouse monoclonal RhoA antibody (26C4) and rabbit polyclonal Rho kinase antibody (C9) were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif). Sildenafil was purchased from Pfizer (Sandwich, UK), and the Rho kinase inhibitor Y-27632, synthesized by Lesieur (Lille University), was a gift from Institut International de Recherche Servier (Courbevoie, France). All other reagents were purchased from Sigma (Saint Quentin Fallavier).

Statistics

All results are expressed as mean ±SEM, and n is the sample size. In experiments with comparison of two conditions, a nonpaired Student’s t test was used. Differences among multiple groups were tested with ANOVA (one-way ANOVA, Fisher’s test). Data were considered statistically significant when P<0.05. Concentration-response curves were fitted to a logistic equation using Origin software (Dipsi).

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

Results

Chronic Hypoxia-Induced Histological Remodeling of the Pulmonary Artery

Hematocrit was increased in rats maintained in the hypobaric chamber for 15 days, attesting the hypoxic condition, and the rise in the RV/LV + S ratio in CH rats was consistent with the development of PHT (Table 1). Extralobar pulmonary arteries

<table>
<thead>
<tr>
<th>TABLE 1. Physiological Parameters</th>
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<tr>
<td>Control</td>
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</tr>
<tr>
<td>Hematocrit, %</td>
</tr>
<tr>
<td>RV/LV + S ratio</td>
</tr>
<tr>
<td>[NOₓ⁻]%, control</td>
</tr>
</tbody>
</table>

Values are mean ±SEM with n=4–6.
*Normoxia vs hypoxia  P<0.001.
†Hypoxia vs hypoxia + sildenafil P<0.01.
of rats maintained in CH for 15 days underwent major morphological alterations attested by a significant increase in the media thickness and area in the absence of change in the thoracic aorta (Table 2). These alterations are associated with histological remodeling, including an increase in the elastin and collagen content without modification of the elastin to collagen ratio (Table 2). Remodeling also affected small pulmonary arteries, which showed medial thickening similar to that classically described in response to CH.26,27

Chronic Hypoxia Alters the Contractile Properties of the Pulmonary Artery

Concentration-dependent contractions were recorded in response to KCl, ET-1, NA, and U46619 in aorta and pulmonary artery rings from normoxic and CH rats. In aorta rings, the parameters of contractile responses were identical in normoxic and CH rats for each agonist tested (not shown). In pulmonary artery rings, contractions induced by KCl in CH rats were similar to those obtained in pulmonary arteries from normoxic and CH rats (40.8±5.9% versus 382.0±3.1% in control, n=4, P<0.001) without significant change in the EC50 (99.2±10.6 versus 117.0±18.7 nmol/L in control, n=4, P>0.05; Figure 1D). These results indicate that alteration of the contractile properties of the pulmonary artery induced by CH depended on the agonist used. Contractions induced by KCl that depend on rise in intracellular Ca2+ concentration were not affected by CH. Contractions induced by ET-1 and NA that involve both pathways of sensitization of contractile proteins14 were reduced by 35% and 37% by CH, respectively. Contractile response induced by U46619 that nearly exclusively involves an increase in Ca2+ sensitivity of the contractile apparatus14 was inhibited by 70% by CH. It could thus be hypothesized that CH alters the contractile properties of pulmonary arteries through the inhibition of agonist-mediated Ca2+ sensitizing mechanisms.

### Table 2. Histomorphometric Parameters

<table>
<thead>
<tr>
<th></th>
<th>Pulmonary Artery</th>
<th>Aorta</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media wall thickness, μm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normoxia</td>
<td>37.36±2.31</td>
<td>102.7±1.33</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>74.45±5.62*</td>
<td>100.7±1.97</td>
</tr>
<tr>
<td>Hypoxia+sildenafil</td>
<td>46.35±3.85†</td>
<td>103.0±2.18</td>
</tr>
<tr>
<td>Media, 10^3 μm^2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normoxia</td>
<td>294±12</td>
<td>597±53</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>616±23*</td>
<td>625±27</td>
</tr>
<tr>
<td>Hypoxia+sildenafil</td>
<td>370±10†</td>
<td>586±68</td>
</tr>
<tr>
<td>Elastin content, %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normoxia</td>
<td>10.67±2.46</td>
<td>18.4±1.5</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>17.44±1.31*</td>
<td>17.6±0.9</td>
</tr>
<tr>
<td>Hypoxia+sildenafil</td>
<td>10.99±1.68†</td>
<td>17.9±1.4</td>
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<tr>
<td>Collagen content, %</td>
<td></td>
<td></td>
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<tr>
<td>Normoxia</td>
<td>8.63±1.31</td>
<td>14.8±0.7</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>13.41±0.99*</td>
<td>14.5±1.1</td>
</tr>
<tr>
<td>Hypoxia+sildenafil</td>
<td>9.01±1.20†</td>
<td>14.2±1.6</td>
</tr>
<tr>
<td>Elastin/collagen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normoxia</td>
<td>1.24±0.09</td>
<td>1.24±0.02</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>1.30±0.11</td>
<td>1.21±0.08</td>
</tr>
<tr>
<td>Hypoxia+sildenafil</td>
<td>1.22±0.14</td>
<td>1.26±0.15</td>
</tr>
</tbody>
</table>

Values are mean±SEM with n=6.
*Normoxia vs hypoxia P<0.0001.
†Hypoxia vs hypoxia+sildenafil P<0.0001.
Chronic Hypoxia Abolishes Rho Kinase–Mediated Ca\(^{2+}\) Sensitization

Ca\(^{2+}\)-dependent contractions and Ca\(^{2+}\) sensitization of contractile proteins could be independently evoked in \(\beta\)-escin–permeabilized smooth muscle strips. Ca\(^{2+}\)-dependent contractions were induced by gradual increase in Ca\(^{2+}\) concentration (submaximal pCa 8 to maximal pCa 4.5), and Ca\(^{2+}\) sensitization was evoked by addition of GTP\(\gamma\)S. The Ca\(^{2+}\) sensitization appears as a leftward shift of the pCa-tension relationship. In \(\beta\)-escin–permeabilized pulmonary artery strips from normoxic rats, GTP\(\gamma\)S induced an increase in the Ca\(^{2+}\) sensitivity of contractile proteins, illustrated by an increase in the pCa\(_{50}\) from 6.10\(\pm\)0.01 (n=8) to 6.68\(\pm\)0.06 (\(P<0.001\), n=8) (Figure 1E). This shift in the pCa-tension relationship was completely abolished in the presence of the Rho kinase inhibitor Y-27632 (10 \(\mu\)mol/L), indicating that the GTP\(\gamma\)S-induced Ca\(^{2+}\) sensitization in pulmonary artery was exclusively mediated by the RhoA/Rho kinase pathway (Figure 1E). The pCa-tension relationship in permeabilized pulmonary artery strips from CH rats was similar to that of controls (pCa\(_{50}\), 6.09\(\pm\)0.01; \(P>0.1\); n=6) (Figure 1F). However, the GTP\(\gamma\)S-induced Ca\(^{2+}\) sensitization was completely lost in pulmonary arteries from CH rats (pCa\(_{50}\), 6.11\(\pm\)0.02; n=6; \(P>0.1\) compared with CH in the absence of GTP\(\gamma\)S), and Y-27632 had no effect (Figure 1F). These results are consistent with an inhibitory effect of CH on Ca\(^{2+}\) sensitization in pulmonary artery smooth muscle and suggest that CH mediates inhibition of the RhoA/Rho kinase signaling pathway.

Chronic Hypoxia Induces a Loss of RhoA in the Pulmonary Artery

The expression of RhoA in pulmonary artery from rats exposed to CH for 4 to 15 days was analyzed by immunoblotting. CH induced a time-dependent loss of RhoA expression, which was already significant after 4 days and almost complete after 15 days (Figure 2A). Reduction of RhoA expression at 15 days was also detected in CH rat lung protein extracts (68\(\pm\)7% of control, n=3), suggesting that the downregulation of RhoA was not restricted to extralobar arteries but also affected small pulmonary arteries. Examination of the abundance of RhoA mRNA by quantitative RT-PCR in the pulmonary artery revealed that the CH-induced decrease in RhoA expression was associated with reduction in RhoA mRNA level (Figure 6A). In contrast to that observed in pulmonary artery, RhoA expression in aorta remained unchanged during exposure to CH (Figure 2B). Expression of Rho kinase, which is the RhoA target responsible for RhoA-mediated Ca\(^{2+}\) sensitization of smooth muscle contraction,\(^{14}\) was not modified by CH, either in the pulmonary artery or in the aorta (Figure 2C). These results suggest that CH-induced inhibition of RhoA-dependent Ca\(^{2+}\) sensitization of pulmonary artery smooth muscle was attributable to CH-induced loss of RhoA expression. However, direct exposure of pulmonary artery smooth muscle cells to hypoxia (2% \(O_2\)) did not decrease RhoA mRNA level (102.7\(\pm\)1.8% and 101.6\(\pm\)1.1% of control [n=4] after 8 and 15 hours of hypoxia, respectively). This result suggests that the decrease in RhoA expression in the pulmonary artery from rats exposed to CH was not directly attributable to hypoxia but involved a secondary mechanism that was absent in cultured cells.

Elevated Intraluminal Pressure Does Not Affect RhoA Expression

Exposure to CH induces a sustained PHT. We therefore assessed whether the decrease in RhoA expression in the pulmonary artery of rats exposed to CH in vivo could be a consequence of the rise in blood pressure. RhoA expression was analyzed in arterial segments pressurized at normal or hypertensive levels for 1 to 3 days. As shown in Figure 3, RhoA expression was not affected by the increase in intraluminal pressure and remained similar to that detected in arteries freshly removed from animals, used as a reference for RhoA protein content in vivo. Thus, the decrease in RhoA expression observed in the pulmonary artery of rats exposed to CH could not be attributed to the rise in pulmonary artery pressure.

Sildenafil Prevents Chronic Hypoxia-Induced Loss of RhoA and RhoA-Mediated Ca\(^{2+}\) Sensitization

CH results in an impaired production/biodisposibility of NO in pulmonary arteries.\(^{28}\) Altered NO signaling was confirmed.
by the reduction of the NO\textsuperscript{x} concentration detected in the plasma from the left ventricle of rat exposed to CH for 15 days compared with normoxic rats (Table 1). This change was associated with a decrease in the maximal endothelium-dependent cholinergic relaxation induced by CCH in the pulmonary artery of CH rats (Figure 4A) without alteration of the EC\textsubscript{50} (16.9±1.3 \mu mol/L in CH versus 19.4±2.1 \mu mol/L in controls; n=5, P>0.05) (Figure 4A). Endothelium-independent relaxation to SNP was not significantly different between the two groups of rats (Figure 4C). None of these changes were found in thoracic aorta, indicating that CH, or indirectly the resulting pulmonary hypertension, specifically altered the NO/cGMP pathway in pulmonary artery (Figures 4B and 4D).

We next assessed whether the effect of CH on RhoA expression and RhoA-dependent Ca\textsuperscript{2+} sensitization in pulmonary arteries was related to alteration of the NO/cGMP signaling pathway. For this purpose, we used sildenafil, an orally active, potent, and selective inhibitor of the type 5 phosphodiesterase. Rats treated orally with sildenafil (25 mg/kg per day) throughout 15 days of exposure to CH exhibited a significant reduction in right ventricular hypertrophy (RV/LV+S ratio, Table 1) and an inhibition of pulmonary vascular remodeling (Figure 5A; Table 2) despite a reduction in NO\textsuperscript{x} concentration similar to that of nontreated CH rats (Table 1). The maximal endothelium-dependent cholinergic relaxation in pulmonary artery of sildenafil-treated CH rats remained reduced similarly to that of nontreated CH rats (27.3±3.1\%: n=4, P>0.05). Sildenafil had no effect in the aorta and pulmonary arteries of normoxic rats and in the aorta of CH rats (Table 2). Our results are thus in agreement with previous observations showing that sildenafil reduces pulmonary arterial pressure in patients with PHT and pulmonary vascular remodeling in mice exposed to CH.\textsuperscript{29,30}

Concentration-response curves to contracting agonists show that sildenafil prevented the decrease in reactivity induced by CH in pulmonary artery (Figure 1). Indeed, the maximal amplitude of the responses to U46619 obtained in sildenafil-treated CH rats (153.8±3.5\%, n=4) was signifi-
The results of the present study show that CH induces downregulation of RhoA expression and RhoA/Rho kinase-mediated Ca\(^{2+}\) sensitization in the pulmonary artery, which in turn is responsible for a decreased response to contracting agonists. These changes of RhoA expression and RhoA-dependent functions are completely prevented by the oral administration of sildenafil, indicating a major role of the NO/cGMP pathway in CH-induced alteration of RhoA signaling in the pulmonary artery.

Although surprising, the evidence for a decrease in the responses of pulmonary arteries to contracting agonists has been previously shown to occur in animal models of PHT. Decreased reactivity to angiotensin I and II has been described in main and intralobar arteries from rats exposed to hypoxia for 1 week.\(^{35}\) Similarly, exposure to hypoxia for 2 weeks leads to reduction in the main pulmonary artery contractile response to ET-1 and angiotensin II.\(^{12}\) In addition, in a rat model of PHT induced by left ventricular infarction, a marked decrease in contractile response to NA occurred in the pulmonary artery, associated with a decreased production of NO.\(^{32}\) The mechanisms leading to this reduced reactivity had not been investigated. However, because this effect was not restricted to a specific type of agonist or membrane receptor, it has been suggested that altered regulation of the Ca\(^{2+}\) sensitivity of the contractile apparatus might be involved.\(^{32}\) Ca\(^{2+}\) sensitization mediated by RhoA and its target Rho kinase is recognized as the major determinant of the sustained rise in tension induced by vasoconstrictors in arterial smooth muscle.\(^{16-18}\) In this study, we demonstrate that RhoA/Rho kinase–mediated Ca\(^{2+}\) sensitization in the pulmonary artery is abolished by CH. We observed that the reduced contractility induced by CH varied quantitatively between agonists used, in accordance with the relative contribution of Rho kinase–mediated Ca\(^{2+}\) sensitization to agonist-mediated contractions.\(^{14}\) This correlation suggests that inhibition of the RhoA/Rho kinase–mediated Ca\(^{2+}\) sensitization is the key process whereby agonist-mediated contraction is reduced by CH in the pulmonary artery. However, we cannot rule out the involvement of other accessory mechanisms, including changes in Ca\(^{2+}\) signaling and ion channel expression or modification of receptor density.

Data are now accumulating regarding the involvement of altered Rho protein expression or activity in arterial disorders such as hypertension, atherosclerosis, and restenosis.\(^{16,19-22}\) Although Rho protein expression or activity has not been directly investigated, in vitro pharmacological studies using the Rho kinase inhibitor Y-27632 have suggested that in response to acute hypoxia, pulmonary vasoconstriction recorded in isolated rat lung or isolated small pulmonary arteries and myosin light chain phosphorylation measured in cultured pulmonary artery smooth muscle cells involved Rho kinase.\(^{33,34}\) Our results show that the amount of RhoA is markedly decreased in pulmonary arteries and lungs of CH rats, inferring that the downregulation of Rho kinase–mediated Ca\(^{2+}\) sensitization induced by CH is attributable to the loss of RhoA expression. Therefore, contrary to that observed in acute response to hypoxia, our data do not support a role for Rho kinase–dependent vasoconstriction in the sustained...
PHT induced by CH. This interpretation is in agreement with the absence of any effect of Y-27632 (30 mg/kg per day), administered throughout the entire duration of exposure to CH, on pulmonary artery remodeling or reactivity and on right ventricular hypertrophy (not shown). In contrast, sildenafil treatment, which strongly reduced right ventricular hypertrophy and pulmonary artery remodeling in CH rats, prevented the CH-induced decrease of RhoA mRNA and protein expression and preserved RhoA/Rho kinase–mediated Ca\(^{2+}\) sensitization. CH-induced PHT is reduced by sildenafil treatment,\(^{35}\) raising the possibility that the observed changes in RhoA expression in CH- and sildenafil-treated CH rats could be secondary to modifications of vascular intraluminal pressure. However, our present results showing that increased intraluminal pressure alone has no effect on RhoA expression suggest that the downregulation of RhoA in pulmonary artery of CH rats was not attributable to the development of PHT. This observation is in agreement with a recent study reporting that arterial expression of RhoA in different hypertensive rat models remained at normotensive levels.\(^{36}\)

Taken together, the present data demonstrate a major role of the NO/cGMP pathway in the modulation of RhoA expression by CH. This agrees with our recent work demonstrating that cGMP-dependent kinase positively regulates RhoA expression through mechanisms involving both cGMP-dependent kinase–mediated regulation of RhoA protein stability and cGMP-dependent kinase–mediated rhoA transcription.\(^{37}\) Also, in N-acetyl-L-arginine–treated rats, chronic inhibition of NO synthesis induced a strong decrease in RhoA mRNA and protein expression in aorta and pulmonary artery, associated with inhibition of RhoA-mediated Ca\(^{2+}\) sensitization.\(^{37}\) Modulation of RhoA expression thus seems to be a regulatory mechanism that controls the capability of cells to respond to external stimuli. Indeed, the reduction of RhoA/ Rho kinase–dependent Ca\(^{2+}\) sensitization associated with the downregulation of RhoA expression indicates that the level of RhoA expression is a limiting factor of RhoA-dependent functions. In addition to contraction, RhoA regulates several processes in vascular smooth muscle cells, including migration, proliferation, gene transcription, and differentiation.\(^{38}\) Consequently, a change in RhoA expression in pulmonary artery smooth muscle may lead to phenotype modulation, abnormal gene expression, and defective response to external stimuli and so participate in the pulmonary arterial wall remodeling induced by CH. The NO-dependent regulation of RhoA expression therefore might thus represent a crucial component of the determinant action of NO on the structure and function of the vessel wall in normal and pathological conditions, where NO biodisponibility is altered.\(^{38}\)

In summary, the data presented herein indicate that CH induces the loss of RhoA expression and RhoA/Rho kinase–mediated Ca\(^{2+}\) sensitization of contraction in the pulmonary artery. These changes are completely prevented by sildenafil, indicating a major role for the NO/cGMP pathway in the CH-induced alteration of RhoA signaling. Therefore, paradoxically, stimulation of the cGMP pathway restores normal contractile properties of pulmonary artery smooth muscle. Taken together, our results also suggest that the sustained PHT induced by CH is more likely to be attributable to pulmonary artery remodeling than to increased vascular reactivity and that the beneficial effect of sildenafil is probably attributable more to its effect on the vessel wall structure than to a direct vasodilator action. Additional studies are now required to understand the consequences of CH-induced loss of RhoA expression in pulmonary artery smooth muscle and their roles in the development of PHT associated with CH.

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Materials and Methods

Animals

All experiments were conducted in accordance with institutional guidelines for the care and use of laboratory animals. Adult male Wistar rats (250 g) were separated into two groups. The normoxic rats were housed in room air at a normal atmospheric pressure (760 mmHg). The hypoxic rats were housed in a hypobaric chamber (Vacucell 111 L, Medcenter, Munich, Germany) for 15 days. The pressure in the chamber was reduced to 380 mmHg and the chamber was continuously flushed with room air to prevent the accumulation of CO₂, NH₃ and H₂O. The hypobaric pressure was maintained 24h/day except when the chamber was opened for 10-30 min twice a week to clean the cages and replenish the food and water. All rats were exposed to a 12:12-h light-dark cycle. Pulmonary hypertension was assessed by measuring the weight ratio of right ventricle (RV) to left ventricle plus septum (LV+S) (RV/LV+S ratio). At completion of the exposure, the aorta and extralobar pulmonary arteries were removed and dissected to remove the adventitial layer. Tissues were then prepared as indicated for contraction measurements, Western blot analysis, RNA extraction or morphological analysis.

NOₓ⁻ assay

Left ventricular plasma NOₓ⁻ concentration was determined by spectrophotometric analysis as described previously.¹ Plasma samples were diluted 1:10 with phosphate buffer. 50 μL of a stock NADPH solution (0.8 μg NADPH/mL phosphate buffer) and 10 μL of a stock nitrate reductase solution (5 units nitrate reductase/mL phosphate buffer) were added to 500 μL of diluted plasma. After incubation for 3 hours at room temperature, Greiss reagent was added and incubated for 10 min at room temperature. The absorbance of plasma samples was
measured at 546 nm. A standard curve was prepared by addition of NaNO₃ to the phosphate buffer.

**Tension measurements in intact fibers**

The aorta and extralobar pulmonary arteries (main and first branch) were collected in physiological saline solution (PSS, in mmol/L; 130 NaCl, 5.6 KCl, 1 MgCl₂, 2 CaCl₂, 11 glucose, 10 Tris, pH 7.4 with HCl) cleaned of fat and adherent connective tissue, and cut in rings. When indicated, the endothelium was carefully removed by gently rubbing the intimal surface with the tip of small forceps. Rings were then suspended under isometric conditions and connected to a force transducer (Pioden controls Ltd, Canterbury, UK) in organ baths filled with Krebs-Henseleit solution (in mmol/L: 118.4 NaCl, 4.7 KCl, 2 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 25 NaHCO₃, 11 glucose) maintained at 37°C, and equilibrated with 95% O₂-5% CO₂. The preparations were initially placed under a resting tension of 1500 mg, left to equilibrate for 1 h and washed at 20 min intervals. Cumulative concentration-response curves were constructed in response to KCl, ET-1, noradrenaline (NA) and the thromboxane A₂ receptor agonist U46619. Amplitude of the contraction was expressed as a percentage of the maximal KCl-induced contraction. The amplitude of relaxation was expressed as percentage of the maximal amplitude of contraction induced by phenylephrine recorded before carbachol (CCH) or sodium nitroprusside (SNP) application.

**Isometric tension measurement in skinned fibers**

Small muscle strips (approximately 200 µm wide and 4 mm long) were isolated from the media of extralobar pulmonary arteries and tied at each end with a single silk thread to the tips of two needles, one of which was connected to a force transducer (AE 801, SensoNor, Horten, Norway). Strips were placed in a well on a bubble plate filled with PSS and stretched to about
1.3 resting length. The solution was rapidly changed by sliding the plate to an adjacent well. After measuring the ontraction evoked by a high-K⁺ solution, strips were incubated in the normal relaxing solution (in mmol/L: 85 KCl, 5 MgCl₂, 5 Na₂ATP, 5 creatine phosphate, 2 EGTA, 20 Tris-maleate, pH 7.1 at 25°C with KOH) for few minutes, followed by treatment with β-escin (50-70 µmol/L) in the relaxing solution for 35 min at 25 °C as previously described.² The skinned muscle strip was then washed several times with fresh relaxing solution containing 10 mM EGTA. Calmodulin (1.5 µmol/L) was added to the bathing solutions throughout the experiments. Tension developed by permeabilized muscle strips was measured in activating solutions, containing 10 mmol/L EGTA and a specified amount of CaCl₂ to give a desired concentration of free Ca²⁺.²

**Western blot analysis**

The lungs, endothelium-denuded aorta and pulmonary arteries were rapidly frozen in liquid nitrogen and homogenized in lysis buffer containing (in mmol/L) 20 Hepes-NaOH, 10 KCl, 10 NaCl, 5 MgCl₂, 1 DTT and Complete (Boehringer, 1 tablet /50 mL). Nuclei and unlysed cells were removed by low speed centrifugation (10,000 g, for 15 min at 4°C). Protein concentration of the supernatant was measured and adjusted, then Laemmli sample buffer was added and equal amounts of protein were loaded in each lane of 12% polyacrylamide/SDS gels, which were then electrophoresed and transferred to nitrocellulose. The amount of proteins was checked by staining with ponceau red. Before immunoblotting, the membrane was blocked with 50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.1% tween, 5% non-fat milk for 1 h at room temperature. Expression of RhoA and Rho kinase was analyzed using mouse monoclonal anti-RhoA antibody or with goat polyclonal anti-Rho kinase I antibody, respectively, then revealed with horseradish peroxidase conjugated secondary antibodies. Signal from immunoreactive bands was detected by ECL (Amersham Pharmacia, Orsay,
France), and quantified using QuantityOne (Biorad, Hercules, CA). Equal loading was checked by reprobing the membranes with anti-β-actin antibody.

**Real time RT-PCR**

Total RNA was extracted using TRIzol Reagent (Life Technologies, Cergy Pontoise, France) and reverse transcription was performed according to standard techniques. Quantitative real-time PCR assays were carried out with sequence-specific primers pairs on the iCycler iQ system (Biorad) using intercalation of Sybr Green as fluorescent probe. The Sybr Green Kit (Perkin Elmer Applied Biosystems, Foster City, CA) was used for real-time monitoring of amplification. Results were evaluated by iCycler iQ Real Time Detection System software (Biorad). The expression of GAPDH mRNA was used to normalize the expression of RhoA mRNA. Primers used were as follows: GAPDH (forward: 5’-CCATGCCATCAGCTGCGACT-3’ and reverse: 5’-TGTCATCATTACGTGCAGGTTTC-3’) and RhoA (forward: 5’-GCAGGTTAGTTGCACTTTATGG-3’ and reverse: 5’-CTTGTGTGCGCTCATCATCCGG-3’).

**Pressurized arteries**

Removed rat carotid artery segments were immersed in an organ culture bath filled with Dulbecco's modified Eagle's medium (Gibco BRL, Cergy Pontoise, France) containing antibiotics (penicillin 100 IU/L, streptomycin 100 mg/L, and amphotericin B 10 mg/L) supplemented with 20% FCS (Boehringer Mannheim, Rueil Malmaison, France). Organ culture of arterial segments was carried out under sterile conditions in an incubator containing 5% CO2 at 37°C. The device used for application of intraluminal pressure to vessel segments in organ culture has been described previously. Each arterial segment was connected to a perfusion circuit consisting of a three-port glass reservoir, a peristaltic pump (Masterflex
60648, Cole-Palmer Instrument Co), and a pressure chamber, which permitted the application of a controlled hydrostatic pressure to the intraluminal compartment. The two lateral ports of the glass reservoir were used for input and output of the circulating intraluminal medium, which was the same as the extraluminal medium described above. Arterial segments were maintained in the organ culture system for 1 or 3 days, pressurized at normal (80 mm Hg) or hypertensive levels (150 mm Hg). Segments used for comparison of different pressures were obtained from the same rat and processed simultaneously. Five carotid segments were studied under each experimental condition. Vessels freshly removed from animals and processed for gel electrophoresis and Western blot served as a reference for Rho protein content in vivo.

Morphological Analysis

Pulmonary arteries and aortas from control and hypoxic rats were collected and fixed for 1 h with paraformaldehyde (4% wt/vol). Arteries were embedded in medium for frozen tissue specimens (Tissue-Tek, Sakura Finetechnical Co., Japan). Transverse sections (6 µm thick) were cut with a cryostat and mounted on precleaned glass slides. Sections were stained with Sirius red (Sigma) for collagen fibers and eosin-hematoxylin (Sigma) for nuclei, then analyzed using Metamorph software (Universal Imaging Co., West Chester, PA, USA).
**Chemicals and drugs**

Mouse monoclonal RhoA antibody (26C4) and rabbit polyclonal Rho kinase antibody (C9) were purchased from Santa Cruz Biotechnology (CA, USA). Sildenafil was purchased from Pfizer (Sandwich, UK) and the Rho kinase inhibitor Y-27632, synthesized by Pr. Lesieur (Lille University, France) was a gift from Institut International de Recherche Servier (Courbevoie, France). All other reagents were purchased from Sigma (Saint Quentin Fallavier, France).

**Statistics**

All results are expressed as the mean ± s.e.m, and n is the sample size. In experiments with comparison of two conditions, a non-paired Student’s $t$-test was used. Differences among multiple groups were tested with ANOVA (one-way ANOVA, Fisher's test). Data were considered statistically significant when $P$ was <0.05. Concentration-response curves were fitted to a logistic equation using Origin software (Dipsi, Chatillon, France).

**References**

