Regression of chronic hypoxic pulmonary hypertension by simvastatin

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Girgis RE, Mozammel S, Champion HC, Li D, Peng X, Shimoda L, Tuder RM, Johns RA, Hassoun PM. Regression of chronic hypoxic pulmonary hypertension by simvastatin. Am J Physiol Lung Cell Mol Physiol 292: L1105–L1110, 2007. First published February 2, 2007; doi:10.1152/ajplung.00411.2006.—The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, simvastatin, has been shown to attenuate chronic hypoxic pulmonary hypertension (CHPH). Here, we assess whether simvastatin is capable of inducing regression of established CPHH and explore potential mechanisms of statin effect. Rats (n = 8 in each group) were exposed to chronic hypoxia (10% FIO2) for 2 or 4 wk. Simvastatin treatment (20 mg·kg−1·day−1) commenced after 2 wk of hypoxia, at which time CPHH was fully established, reduced mean pulmonary artery pressure (19 ± 0.5 vs. 27 ± 0.9 mmHg; P < 0.001), the ratio of right ventricular free wall to left ventricular plus septal weight (0.4 ± 0.3 vs. 0.27 ± 0.03; P < 0.001), and medial thickening of small pulmonary arteries (13 ± 0.4 vs. 16 ± 0.4%; P < 0.01) compared with 4-wk hypoxic controls. Supplementation with mevalonate (50 mg·kg−1·day−1) prevented the attenuation of CPHH induced by simvastatin during 2 wk of hypoxia. Because statins are known to inhibit Rho-kinase (ROCK), we determined expression of ROCK-1 and -2 in whole lung by Western blot and ROCK activity by phosphorylation of the myosin-binding subunit (MBS) of myosin phosphatase. Expression of both ROCK-1 and -2 were markedly diminished in simvastatin-treated animals during normoxia and hypoxia (2- and 4-wk exposure (P < 0.01). ROCK activity was increased threefold under hypoxic conditions and normalized with simvastatin treatment (P < 0.001). We conclude that simvastatin attenuates and induces regression of established CPHH through inhibition of HMG-CoA reductase. Inhibition of ROCK expression and activity may be an important mechanism of statin effect.

Rho-kinase; mevalonate; statins

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trophy. The left lung was snap-frozen in liquid nitrogen and subsequently stored at −80°C.

Vascular morphology. The right lung was inflated by tracheal infusion of low melt agarose at an airway pressure of 25 cmH2O and immersed in 4% paraformaldehyde for 48 h. Two midsagittal slices were cut parallel to the hilum, washed in PBS, and stored in 70% ethanol. Six-micrometer-thick paraffin sections were stained with Movat’s pentachrome stain for assessment of medial wall thickness. Circular or quasi-circular arteries (defined as a ratio between short and long diameter of ≥0.7) with an external diameter (ED) of 25–100 μm were analyzed. The perimeter of the vessel was traced around the outer aspect of the external elastic lamina, and diameter was calculated as perimeter/π. The average medial thickness (MT) was taken from four measurements obtained at each quadrant and expressed as 2 × MT/ED. Twenty vessels in consecutive fields were examined for each animal using six samples from each group by an investigator masked for the identity of the experimental groups.

ROCK-1 and -2 Western blot. Western blotting for ROCK-1 and -2 was performed on whole lung homogenates (9) from six animals in each group. Lungs were homogenized using a Polytron in 5 volumes of ice-cold buffer [50 mM Tris-HCl (pH 7.2), 500 mM NaCl, 10 mM MgCl2, 1 mM EDTA, 1% Triton X-100, 0.5% deoxycholic acid, 0.1% SDS, 20 μg/ml each of leupeptin and aprotinin, and 1 mM PMSF] followed by centrifugation at 5,000 g at 4°C for 10 min. Protein concentration was determined with Bicinchoninic Acid (BCA) Protein Assay Kit (Pierce Biotechnology, Rockford, IL). Equal amounts (20 μg) of supernatant protein were separated under denaturing conditions in a 4–20% linear gradient SDS-PAGE gel, followed by blotting of the proteins onto polyvinylidene difluoride membranes. Blots were blocked at room temperature for 1 h in 50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 2% BSA, and 0.1% Tween 20. The membrane was cut to allow separate primary antibody incubations with ROCK-1 or -2 (dilution 1:1,000; BD Transduction Laboratories, Lexington, KY) and β-actin antibodies (dilution 1:10,000; Abcam, Cambridge, MA) overnight at 4°C. Membranes were then washed at room temperature and incubated with an anti-mouse IgG conjugated to horseradish peroxidase (dilution 1:1,000; Pierce Biotechnology) for 1 h at room temperature. Immunoreactive protein bands were detected with ECL (Amersham, Piscataway, NJ). Densitometry was performed to quantify the amount of protein, and the ratio of ROCK-1 or ROCK-2-to-β-actin was calculated. Fold induction was determined by setting the ratio in normoxic animals equal to 1.

ROCK activity assay. ROCK enzymatic activity of homogenized lung samples was determined with a commercially available nonquantitative immunoassay kit (Rho-kinase assay kit; Cyclex, Nagano, Japan) according to the manufacturer’s instructions (11). Plates are precoated with a substrate that corresponds to the COOH terminus of recombinant MBS, which contains a threonine residue that may be phosphorylated by myotonic dystrophy protein kinase (DMPK) family members, including ROCK-1, ROCK-2, and DMPK. The detector antibody specifically detects only the phosphorylated form of Thr696 on MBS. Fold change in enzyme activity was determined by setting the absorbance of normoxic controls as 1.

Immunohistochemistry for PCNA after 3 days of hypoxia. In a separate experiment, rats (n = 4 per group) were exposed to 3 days of hypoxia or normoxia with or without 20 mg·kg−1·day−1 simvastatin. Lung sections were assessed for PCNA by immunohistochemistry. After deparaffinization, the slides were boiled in 1% citric buffer for 5 min and then incubated with anti-PCNA antibody (1:250 dilution; DAKO, Carpinteria, CA) diluted in 5% goat serum in PBS for 6 h. Following washing in PBS, the secondary goat anti-mouse IgG antibody conjugated with biotin (1:200 dilution; Vector Lab, Burlingame, CA) was applied and incubated for 1 h at room temperature followed by incubation with avidin-biotin complex (1:200 dilution; Vector Lab). A Vector 3,3-diaminobenzidine (DAB) kit (Vector Lab) was used following the manufacturer’s instructions. After washing in water, the slides were counterstained with hematoxylin and mounted with Permount (Fisher, Pittsburgh, PA).

One hundred vessels with ED 25–100 μm were assessed by an investigator masked to the identity of each animal, and the percentage positive for PCNA staining was determined.

Statistical analysis. Data are expressed as means ± SE. Comparisons between groups were performed with one-way ANOVA and Tukey’s multiple comparisons test or Kruskal-Wallis test, followed by Dunn’s multiple-comparison test, as appropriate. Comparisons between the two groups in the 28-day experiment and for PCNA staining at 4 days were compared with unpaired t-test or Mann-Whitney U test, as appropriate. A P value less than 0.05 was considered significant. GraphPad Prism software (San Diego, CA) was utilized for all analyses.
RESULTS

*Attenuation of CPH by simvastatin.* Consistent with our (6) previous results with intraperitoneal injection, oral administration of simvastatin markedly attenuated hypoxia-induced elevations in pulmonary artery pressure, right ventricular hypertrophy (Fig. 1), and medial wall thickening of small pulmonary arteries (Figs. 2 and 3). We also confirmed our previous observation that simvastatin suppressed hypoxia-induced polycythemia (Table 1). Supplementation with mevalonate, a metabolite that results from the action of HMG-CoA reductase, completely reversed the reduction in right ventricular hypertrophy and vascular remodeling and partially abrogated the simvastatin treatment effect on pulmonary artery pressure and hematocrit (Figs. 1–3, Table 1). Systemic arterial pressure was comparable among all groups, and weight gain was not altered by either simvastatin or mevalonate treatment (Table 1).

*Regression of established pulmonary hypertension.* Treatment initiated once pulmonary hypertension was fully established after 2 wk of chronic hypoxia induced regression of pulmonary artery pressure, right ventricular hypertrophy, and medial wall thickness, even with ongoing hypoxia exposure, to values comparable to those observed in the attenuation experiment (Table 2).

*ROCK expression.* Western blot analysis of lung tissue demonstrated a marked reduction in the expression of both ROCK-1 and ROCK-2 isoenzymes by simvastatin under normoxic as well as hypoxic exposure in both the attenuation and regression experiments (Fig. 4). There was a small, ~1.2-fold induction of ROCK-1 in both the 2- and 4-wk hypoxia groups relative to normoxia that did not achieve statistical significance with ANOVA testing. Mevalonate supplementation completely reversed the changes in ROCK-1 and -2 expression induced by simvastatin, whereas mevalonate alone induced no changes relative to hypoxic controls.

*ROCK enzyme activity.* The enzymatic activity of ROCK was increased more than threefold after exposure to 2 or 4 wk of hypoxia relative to normoxia (Fig. 5). Simvastatin treatment normalized this activity in both the attenuation and regression experiments but had no effect under normoxic conditions. However, the optical density of the normoxic control samples was just above that recorded in blank wells with buffer alone. In contrast to the observations with ROCK protein expression, mevalonate supplementation only partially restored enzyme activity in hypoxia-simvastatin-treated animals. Post-ANOVA multiple-comparison testing showed no significant difference between HS and HSM. Mevalonate alone had no effect on enzymatic activity during hypoxia. The specificity of the assay

![Fig. 2. Representative photomicrographs (×400; Movat’s pentachrome stain) of small pulmonary arteries after 14 days of normoxia (A), hypoxia (B), hypoxia plus simvastatin (C), and hypoxia plus simvastatin plus mevalonate (D). Bar represents 50 μm.](http://ajplung.physiology.org/)

![Fig. 3. Comparison of medial wall thickening/external diameter (MWT/ED) of pulmonary arteries 25–100 μm ED among 4 groups of rats; n = 120 vessels per group.](http://ajplung.physiology.org/)
was confirmed by the normalization of activity in hypoxic samples with the addition of 10 μM ROCK inhibitor Y-27632 (data not shown).

**PCNA staining after 3 days of hypoxia exposure.** Normoxic controls and normoxic-simvastatin-treated rat lungs demonstrated faint staining in 13% of vessels. Exposure to hypoxia for 72 h induced intense expression of PCNA staining in the walls of small pulmonary arteries with positive expression in 53% of vessels. Simvastatin treatment reduced the percentage of positively stained vessels to 34% (P < 0.05). Positive vessels, in general, demonstrated faint staining compared with hypoxia controls (Fig. 6).

**DISCUSSION**

Statin therapy has now been shown to be effective in several animal models of pulmonary hypertension. We (6) have previously reported the attenuation of pulmonary hypertension, right ventricular hypertrophy and vascular remodeling induced by 2 wk of hypoxia exposure in rats treated with simvastatin given by intraperitoneal injection. Murata et al. (15) made similar observations with oral fluvastatin. Nishimura and colleagues demonstrated attenuation (17) and regression (i.e., improvement of established pulmonary hypertension; Ref. 18) of pulmonary arterial neointimal formation in pneumonectomized rats injected with monocrotaline treated with simvastatin by gavage. More recently, Taraseviciene-Stewart and co-workers (23) showed that simvastatin prevented the progression of pulmonary vascular disease in a model of severe pulmonary hypertension induced by the combination of chronic hypoxia and injection of SU-5416, a VEGF receptor-2 antagonist.

In this study, we demonstrate that 2 wk of oral simvastatin therapy induces reduction of established CPHH, as evidenced by the significant reduction in pulmonary artery pressure, right ventricular hypertrophy, and medial vascular wall thickness. Moreover, we conclusively show that the mechanism(s) of action of simvastatin in CPHH is dependent on HMG-CoA reductase inhibition since supplementation with mevalonate prevented the attenuation of pulmonary hypertension.

The synthesis of mevalonate is the rate-limiting step in cholesterol formation and also serves as the precursor for isoprenoid intermediates such as geranylgeranyl, which binds to the Rho family of molecules. This allows membrane translocation and GTPase signaling activity with consequent activation of ROCK and other downstream targets (2). Indeed,

<table>
<thead>
<tr>
<th>n = 8 per group</th>
<th>Normoxia</th>
<th>Normoxia + Simva</th>
<th>Hypoxia</th>
<th>Hypoxia + Simva</th>
<th>Hypoxia + Simva + Mevalonate</th>
<th>Hypoxia + Mevalonate</th>
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<tr>
<td><strong>Mean Arterial Pressure</strong></td>
<td>97 ± 1</td>
<td>98 ± 1</td>
<td>102 ± 1</td>
<td>99 ± 1</td>
<td>98 ± 1</td>
<td>99 ± 1</td>
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<tr>
<td><strong>Hematocrit</strong></td>
<td>43 ± 4</td>
<td>46 ± 1</td>
<td>57 ± 1</td>
<td>49 ± 1*</td>
<td>52 ± 1#</td>
<td>54 ± 1</td>
</tr>
<tr>
<td><strong>Weight Change, g</strong></td>
<td>88 ± 4</td>
<td>73 ± 8</td>
<td>44 ± 6</td>
<td>57 ± 5</td>
<td>50 ± 4</td>
<td>43 ± 4</td>
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Values are means ± SE. Simva, simvastatin. *P < 0.01 vs. hypoxia; #P < 0.05 vs. hypoxia; and $P < 0.01$ vs. hypoxia + simva.

**Table 2.** Effects of 2 wk of simvastatin treatment begun after 2 wk of hypoxia alone

<table>
<thead>
<tr>
<th></th>
<th>PAP</th>
<th>RV/LV + S</th>
<th>Hematocrit</th>
<th>MWT</th>
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<tr>
<td>4-wk Hypoxia</td>
<td>27 ± 1</td>
<td>0.54 ± 0.03</td>
<td>58 ± 1</td>
<td>0.162 ± 0.004</td>
</tr>
<tr>
<td>2-wk H + 2-wk HS</td>
<td>19 ± 0.5</td>
<td>0.41 ± 0.03</td>
<td>52 ± 1</td>
<td>0.131 ± 0.004</td>
</tr>
</tbody>
</table>

Values are means ± SE. H, hypoxia; HS, hypoxia plus simvastatin; PAP, pulmonary artery pressure; MWT, medial wall thickness; RV/LV + S, right ventricular free wall to left ventricular plus interventricular septal weight. N = 8 per group. $P < 0.01$ for all comparisons.

Fig. 4. Bar graphs (top) comparing mean (error bars represent SE) relative density of Rho-kinase-1 (ROCK-1; A) and ROCK-2 (B) protein expression relative to β-actin and normalized to normoxic control group. Representative immunoblots are shown at bottom. H4, 4-wk hypoxia controls; HS4, 2 wk of hypoxia alone plus 2 wk of hypoxia plus simvastatin treatment. n = 8 in each group.
many of the beneficial effects of statins in cardiovascular disease have been shown to be mediated through mevalonate-dependent inhibition of the Rho-ROCK pathway (14). A reduction in serum cholesterol is unlikely to explain the effect of simvastatin on pulmonary hypertension. Normal rats are resistant to the cholesterol-lowering action of statins (3). Moreover, serum cholesterol did not change in response to simvastatin therapy in the monocrotaline/pneumonectomy model (17).

Statins possess a host of cholesterol-lowering-independent properties on the vascular wall. Prominent among these is the mevalonate-sensitive inhibition of VSMC proliferation induced by a variety of stimuli (12, 13). Our demonstration of a reduction in PCNA staining of vascular walls by simvastatin 3 days after hypoxic exposure, at which time smooth muscle proliferation is near its peak (20), indicates that this may be an important mechanism in CHPH. Nishimura et al. (18) have similarly reported that simvastatin reduces pulmonary arterial wall PCNA staining in a model of monocrotaline injection plus pneumonectomy. Regression of established disease likely also involves the induction of apoptosis in vascular wall cells, as has been previously shown (18, 23).

A growing body of evidence indicates that ROCKs play an important role in the pathogenesis of several cardiovascular diseases through their effects on vascular tone, actin cytoskeletal organization, cell migration and proliferation, and gene expression and likely represent a key target of statin therapy (19). Chronic administration of ROCK inhibitors has been shown to attenuate the development of pulmonary hypertension and vascular remodeling induced by chronic hypoxia (5, 7, 9), monocrotaline administration (1), and in the Fawn-Hooded rat at altitude (16). We detected a greater than threefold elevation in ROCK enzymatic activity in whole lung homogenates as assessed by the phosphorylation of the MBS of myosin phosphatase. This is consistent with the previous demonstration of enhanced RhoA (10, 16) and ROCK activity (7) in chronic hypoxic rat pulmonary arteries. In contrast, expression of ROCK-1 was only slightly increased by 24% after 2 wk of hypoxia, and no change was observed in the expression of ROCK-2 relative to normoxic. Hyvelin and coworkers (9) reported an ~50% increase in the expression of both isoenzymes after 1 wk of hypoxia.

Treatment with simvastatin normalized the hypoxia-induced increase in ROCK activity. Expression of both ROCK-1 and -2 was also markedly reduced by simvastatin therapy during hypoxic as well as normoxic conditions. The failure to detect a reduction in ROCK activity in simvastatin-treated normoxic animals may have been related to the already low level in normoxic controls. The regulation of ROCK expression has not been clearly defined but appears to involve the transcription factors NF-κB and PKC (8), both of which have been reported to be suppressed by statins (4, 25). Whereas the downregulation of ROCK expression in statin-treated hypoxic animals was completely reversed by mevalonate supplementation, the hypoxia-induced increase in ROCK activity was only partially restored. This may suggest that mechanisms independent of HMG-CoA reductase inhibition may be involved in the suppression of ROCK activity, e.g., allosteric binding to an integrin (24). However, any such action is insufficient to have an effect on pulmonary hypertension and vascular remodeling.

In summary, simvastatin attenuates CHPH through mevalonate-dependent mechanisms. These may include inhibition of VSMC proliferation and downregulation of ROCK expression and activity. Moreover, statin therapy induces regression of established disease and may prove to be a useful adjunct in the management of pulmonary hypertension in humans.

![Fig. 6. Immunohistochemical staining for PCNA after 72 h (3d) of hypoxia and with and without simvastatin treatment showing reduced intensity of positive staining within vascular walls of small pulmonary arteries.](http://ajplung.physiology.org.org)
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GRANTS

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