Attenuation of chronic hypoxic pulmonary hypertension by simvastatin

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Girgis, Reda E., Dechun Li, Xinhua Zhan, Joe G. N. Garcia, Rubin M. Tudor, Paul M. Hassoun, and Roger A. Johns. Attenuation of chronic hypoxic pulmonary hypertension by simvastatin. Am J Physiol Heart Circ Physiol 285: H938–H945, 2003. First published May 15, 2003; 10.1152/ajpheart.01097.2002.—The 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) have been shown to improve multiple normal endothelial cell functions and inhibit vascular wall cell proliferation. We hypothesized that one such agent, simvastatin, would attenuate chronic hypoxic pulmonary hypertension. Male adult Sprague-Dawley rats were exposed (14 days) to normoxia (N), normoxia plus once-a-day administered simvastatin (20 mg/kg ip) (NS), hypoxia (10% inspired O2 fraction) (H), or hypoxia plus simvastatin (HS). Mean pulmonary artery pressure, measured in anesthetized, ventilated rats with an open-chest method, was reduced from 25 ± 2 mmHg in H to 18 ± 1 in HS (P < 0.001) but did not reach normoxic values (12 ± 1 mmHg). Similarly, right ventricular/left ventricular plus interventricular septal weight was reduced from 0.53 ± 0.02 in the H group to 0.36 ± 0.02 in the HS group (P < 0.001). The increased hematocrit in H (0.65 ± 0.02) was prevented by simvastatin treatment (0.51 ± 0.01, P < 0.001). Hematocrit was similar in N versus NS. Alveolar vessel muscularization and medial thickening of vessels 50–200 μM in diameter induced by hypoxia were also significantly attenuated in the HS animals. Lung endothelial nitric oxide synthase (eNOS) protein expression in the HS group was less than in H (P < 0.01) but was similar in N versus NS. We conclude that simvastatin treatment potently attenuates chronic hypoxic pulmonary hypertension and polycythemia in rats and inhibits vascular remodeling. Enhancement of lung eNOS expression does not appear to be involved in mediating this effect. The 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors (statins) have been shown to exert numerous effects on vascular wall function, independent of their cholesterol-lowering effect, that would be expected to have a beneficial impact on the remodeling of pulmonary hypertension (44). For example, statins upregulate endothelial nitric oxide synthase (eNOS) expression and activity (13), increase prostacyclin (40), and reduce endothelin production by endothelial cells (20). Statins are also potent inhibitors of vascular smooth muscle cell growth (30), a prominent feature of human and experimental pulmonary hypertension (10). Many of these actions are a consequence of inhibiting mevalonate synthesis. The latter is the precursor of not only cholesterol but also isoprenoid intermediates required for the activity of Rho and other small G proteins (44).

To determine the possible utility of statin therapy in pulmonary hypertension, we tested the ability of simvastatin to attenuate pulmonary hypertension and pulmonary vascular remodeling in a rat model of chronic hypoxia-induced pulmonary hypertension. We also explored the potential mechanism by assessing the effect of treatment on lung expression of eNOS.

MATERIALS AND METHODS

Experimental animals and treatment. Male adult Sprague-Dawley rats (Hilltop Lab Animals; Scottsdale, PA) weighing between 150 and 250 g were utilized. All animal care and procedures were performed in accordance with institutional guidelines. Four groups of rats studied were the following:

CHRONIC PULMONARY HYPERTENSION is characterized by a component of abnormal pulmonary vasoconstriction and by structural remodeling of the small pulmonary arteries. Both processes lead to a progressive increase in pulmonary vascular resistance, which, when extreme, culminates in right ventricular (RV) failure and death. The syndrome occurs in diverse clinical settings, including lung disease associated with alveolar hypoxia. Endothelial cell injury-dysfunction is considered to be a key factor in the pathogenesis of pulmonary hypertension (3), leading to increased vascular smooth muscle tone, cell proliferation in the vascular wall, and activation of thrombotic mechanisms, all of which participate in the process of remodeling. Currently available therapies have a beneficial clinical effect yet cannot reverse the disease process. Therefore, the response is variable with considerable morbidity and mortality despite therapy (33). Clearly, there is an urgent need for new therapies.

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nromoxic controls (n = 7), hypoxic controls (n = 8), normoxic simvastatin treated (n = 8), and hypoxic simvastatin treated (n = 8). The animals were housed in a Plexiglas chamber open to room air (normoxia groups) or maintained at 10% inspired O₂ fraction (hypoxia groups) for 14 days. Hypoxia was maintained using a ProOx model 350 unit (Reming Bioinstruments; Redfield, NY) that controlled fractional concentration of O₂ in inspired gas by solenoid-controlled infusion of N₂ (Roberts Oxygen; Rockville, MD) balanced against an inward leak of air through holes in the chamber (32). The chamber was opened once daily for drug administration. Water and rat chow were provided ad libitum. The animals were maintained at 20–24°C in a room with a 12:12 h light-dark cycle. Drug-treated rats received a daily intraperitoneal injection of simvastatin (Merck; Whitehouse Station, NJ) at a dose of 20 mg·kg⁻¹·day⁻¹, and the other groups were given an equal volume of vehicle. This dose was chosen based on findings in mice of maximal stroke protection and enhancement of eNOS activity compared with 2 mg/kg (11). Simvastatin was prepared by being dissolved in ethanol then being activated by alkaline hydrolysis to open the lactone ring, forming it to be counted for each form (28). The final concentration used was 4 mg/ml (0.01 M).

Assessment of pulmonary hypertension. After 14 days, the animals were removed from their respective chambers and anesthetized with an intraperitoneal injection of xylazine (10 mg/kg) and ketamine (100 mg/kg). A tracheal cannula was then inserted, and the animals were ventilated with room air with a Harvard rodent ventilator model 683 (Harvard Apparatus; South Natick, MA) that was set at a rate of 90 breaths/min and tidal volume of 8 ml/kg body wt. To measure pulmonary arterial pressure, the chest of the rat was opened via a midline incision. An 18-gauge catheter filled with heparinized saline was inserted through the wall of the right ventricle and advanced into the pulmonary artery. Pressure in the pulmonary artery was measured with a Datascope 2001A (Paramus, NJ). A 3-ml sample of blood was drawn from the left ventricle into EDTA tubes for measurement of hematocrit by centrifugation. The rats were then euthanized by exsanguination, and the heart and lungs were removed en bloc. The heart was dissected and the right ventricular free wall to left ventricular plus interventricular septal [RV/(LV + S)] weight determined. The right lower lobe of the lung was then isolated, was placed in liquid nitrogen, and subsequently stored at −80°C for subsequent Western blot analysis.

Immunohistochemistry and vascular morphology. The remainder of the lung was then inflated by tracheal infusion of 5 ml of paraformaldehyde (4% wt/vol) in 0.1 M phosphate-buffered saline (PBS, pH 7.4). The lung was fixed for 2 h, and three to four sagittal slices were obtained, cutting parallel to the hila. The lung slices were then washed in PBS and stored in 70% ethanol. Paraffin sections (6 μm thick) were then mounted on precleaned glass slides. Lung sections were stained with hematoxylin and eosin and monoclonal α-smooth muscle actin antibody (1:100; Sigma; St. Louis, MO) as previously described (12) for assessment of vascular morphology. Peripheral pulmonary arteries associated with alveolar sacs and ducts were classified as nonmuscular (0–25% of circumference with actin staining), partially muscular (26–75%), and fully muscular (>75% of circumference). Between 50 and 100 vessels were counted for each animal in the two hypoxic groups, the percent medial thickness (%MT) of muscularized arteries measuring 50–200 μm in external diameter (ED) was determined using an Olympus-BHS microscope coupled to an MTI color video camera (DAGE-MTI; Michigan City, IN) and I Cube video grabber board. Measurements were obtained with ImagePro Plus software (Media Cybernetics; Silver Spring, MD) after calibration with an Olympus 0.01-mm calibration slide. Only arteries with a circular or quasicircular outline were examined. The average of three measurements was taken for medial thickness. %MT was calculated as (MT × 2/ED) × 100. A total of 20 arteries in consecutive fields was examined for each animal. All vascular morphology assessments were performed in a blinded fashion.

Lung eNOS. Western blotting for eNOS was performed on whole lung homogenates as previously described (12) from six animals in each group. Briefly, lung homogenates (50 μg of protein/rat) were separated under denaturing conditions in a 4–20% linear gradient SDS-PAGE gel, followed by blotting of the proteins to nitrocellulose (Bio-Rad; Burlingame, CA). 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. Subsequently, blots were incubated with a mouse anti-eNOS monoclonal antibody (dilution 1:1,000; Transduction Laboratories; Lexington, KY) for 1 h at room temperature. Membranes were then washed at room temperature and incubated with horseradish peroxidase (Bio-Rad) for 1 h at room temperature. eNOS immunoreactive protein was detected with enhanced chemiluminescence (ECL, Amersham; Piscataway, NJ) and exposure to film (Hyperfilm-ECL, Amersham). Signal bands were quantified by densitometry (Personal Densitometer, ImageQuant, Molecular Dynamics; Sunnyvale, CA).

Statistical analysis. Data are expressed as means ± SE. Comparisons between groups was performed with one-way ANOVA and Dunnett’s method for post hoc multiple comparisons assuming unequal variances using SPSS software (Chicago, IL). Comparison of percent medial wall thickness between the hypoxia and hypoxia/simvastatin-treated groups was performed with an independent sample t-test. A P value <0.05 was considered significant.

RESULTS

Effect of simvastatin on hemodynamic parameters. The normoxic group gained 127 ± 2 g during the 14-day experiment, whereas the normoxic simvastatin-treated and hypoxic control groups only gained 58 ± 8 and 48 ± 2 g, respectively (P < 0.01 for each group compared with normoxia). The hypoxic simvastatin-treated animals had a minimal weight gain of 12 ± 7 g (P < 0.01 compared with hypoxia alone). No differences were observed in the pulmonary artery pressure, relative right ventricular weight, or hematocrit between normoxia and normoxia/simvastatin-treated animals (Figs. 1–3). Mean pulmonary artery pressure (MPAP) and relative right ventricular weight more than doubled in hypoxic animals compared with normoxic controls (Figs. 1 and 2). Simvastatin treatment reduced this increase in both MPAP and RV/(LV + S) by >50%. As expected, the hematocrit was higher in the hypoxic group compared with normoxia. Surprisingly, simvastatin treatment essentially abolished the polycythemic response to hypoxia (Fig. 3).

Effect of simvastatin on vascular morphology. The alveolar vessels in normoxic animals showed scant staining on smooth muscle α-actin antibody-immunos- tained sections. No differences in vascular morphology were observed between normoxic controls and normoxic/simvastatin-treated rats. In hypoxia-treated an-
animals, there were significant increases in muscularized vessels (Table 1). Treatment with simvastatin was associated with a marked reduction in the muscularization of these arteries induced by hypoxia (Table 1 and Fig. 4). The percent medial wall thickness of arteries 50–200 μm in internal diameter was significantly reduced by simvastatin treatment (25.3 ± 0.7%) compared with hypoxic controls (28.8 ± 0.7%; \( P = 0.003 \)).

**Effect of simvastatin on lung eNOS.** Western blotting of lung homogenates revealed the anticipated increase in eNOS expression induced by hypoxia. No difference in eNOS expression was observed between normoxic controls and normoxia/simvastatin-treated animals. Interestingly, simvastatin treatment attenuated the hypoxic upregulation of eNOS protein (Fig. 5).

### DISCUSSION

Statins, the HMG Co-A reductase inhibitors, have had a dramatic impact on clinical outcomes in patients with coronary artery disease. Several studies have documented benefits unrelated to cholesterol lowering (7, 19) with multiple “pleiotropic” effects on vascular wall function that would be expected to attenuate vascular remodeling (44). These effects stem from their ability to reduce the production of the isoprenoid intermediates farnesyl and geranylglycerol pyrophosphate, compounds that are distal to mevalonate in the cholesterol synthetic pathway. These lipophilic molecules are then covalently bound to Rho and other small G proteins in a posttranslational modification that is essential for attachment of these important signaling proteins to cell membranes and regulators and for their activation of downstream effectors (43).

In this study, we examined the hypothesis that statin treatment could attenuate pulmonary hypertension and vascular remodeling in a rat model of chronic hypoxic pulmonary hypertension. A 14-day duration of hypoxic exposure and concomitant treatment with simvastatin was chosen because maximal effects on right ventricular hypertrophy and vascular remodeling are noted at this time point (22), and most of the pleiotropic actions of statins are detectable within hours to days (20, 26, 41). We found that simvastatin treatment was associated with a marked reduction in pulmonary artery pressure in response to chronic hypoxia. Right

<table>
<thead>
<tr>
<th>Vessel Type</th>
<th>Normoxia, %</th>
<th>Hypoxia, %</th>
<th>Normoxia + Simvastatin, %</th>
<th>Hypoxia + Simvastatin, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fully muscular</td>
<td>6 ± 1</td>
<td>40 ± 3(^a)</td>
<td>7 ± 1</td>
<td>15 ± 3(^b)</td>
</tr>
<tr>
<td>Partially muscular</td>
<td>16 ± 3</td>
<td>25 ± 2</td>
<td>21 ± 2</td>
<td>36 ± 3(^c)</td>
</tr>
<tr>
<td>Nonmuscular</td>
<td>78 ± 4(^d)</td>
<td>35 ± 2</td>
<td>72 ± 3(^e)</td>
<td>49 ± 4</td>
</tr>
<tr>
<td>Total no. of vessels counted</td>
<td>424</td>
<td>513</td>
<td>454</td>
<td>509</td>
</tr>
</tbody>
</table>

Values (means ± SE) reflect the percentage of vessels associated with alveolar spaces and ducts categorized by vessel type in each group; \( n = 7 \) rats for normoxia group and \( n = 8 \) rats for all other groups. \(^a\)\( P < 0.001 \) for hypoxia vs. each of the other groups. \(^b\)\( P < 0.05 \) for hypoxia + simvastatin vs. normoxia. \(^c\)\( P < 0.01 \) for hypoxia + simvastatin vs. normoxia and normoxia + simvastatin. \(^d\)\( P < 0.001 \) for normoxia vs. hypoxia and hypoxia + simvastatin. \(^e\)\( P < 0.01 \) for normoxia + simvastatin vs. hypoxia + simvastatin.
Fig. 4. Representative photomicrographs of rat lung after 14 days of normoxia, normoxia + simvastatin treatment, hypoxia, and hypoxia + simvastatin. Hematoxylin + eosin-stained (H&E, left) sections show a reduction in hypoxia-induced wall thickening by simvastatin treatment. Smooth muscle α-actin (red)-stained sections (right) reveal considerable reduction in hypoxia-induced small vessel muscularization. Arrows indicate vessels. Bars represent a length of 50 μM.
ventricular hypertrophy, as indicated by the RV(LV + S) weight ratio, was also dramatically decreased, indicating a lower pulmonary vascular resistance. Morphological assessment of the pulmonary vasculature revealed considerably less muscularization of small arteries accompanying the alveolar spaces and ducts and decreased medial thickness of medium-sized arteries related to bronchioles. This suggests a reduction in hypoxia-induced vascular smooth muscle cell hyperplasia in response to simvastatin. A recent report demonstrating the dramatic effect of simvastatin on the development of pulmonary hypertension and neointimal proliferation in a monocrotaline plus pneumonectomy rat model (37) is consistent with our data. Additional evidence supporting the potential usefulness of these agents is the abrogation of monocrotaline-induced pulmonary hypertension and vascular remodeling by limonene and sobrerol (45). Simvastatin markedly decreased the levels of eNOS protein as a single 135-kDa band. B: quantitation of eNOS protein signal. y-Axis is in densitometric units. Results are means ± SE; n = 6 rats in each group. *P < 0.01 for hypoxia vs. normoxia and normoxia + simvastatin. †P < 0.05 for hypoxia vs. hypoxia + simvastatin.

Statins have been shown to increase eNOS expression in systemic endothelial cells in vitro (29) and in the systemic circulation of mice (1). Surprisingly, statin treatment in the present study actually decreased eNOS expression in whole lung homogenates of chronically hypoxic rats to levels similar to normoxic controls. No effect of simvastatin treatment on lung eNOS protein levels was observed in normoxic animals. Thus enhanced lung eNOS expression does not appear to account for the attenuation of pulmonary hypertension in our study. Chronic hypoxia is known to increase lung eNOS expression in rats. Whereas some authors (12, 31) contend that it is hypoxia per se that induces eNOS expression, others (4, 38) have provided evidence supporting a more important role for hemodynamic factors. If the latter is the case, the lower pulmonary artery pressure and/or blood viscosity (from lower hematocrit) in the hypoxia plus simvastatin group could have accounted for decreased eNOS expression compared with hypoxia alone. In the study by Nishimura et al. (37), simvastatin treatment was associated with restoration of the reduced eNOS expression observed...
with monocrotaline plus pneumonectomy. Whereas it is clear that chronic hypoxic pulmonary hypertension is associated with increased eNOS expression in rats, the effect of chronic hypoxia on lung eNOS activity is controversial (17). A recent study by Murata and colleagues (35) studied eNOS activity in the isolated pulmonary artery obtained at 1 wk in chronically hypoxic, hypertensive rats. Using a fluorescence microscopic technique to directly visualize the endothelium in situ, the authors demonstrated markedly reduced carbachol-induced endothelial NO production in hypoxic segments compared with normoxia, indicating impaired eNOS enzyme activity. They also showed that eNOS was more tightly coupled with caveolin-1 (a negative regulator of eNOS activity) and was dissociated from heat shock protein (HSP)-90 (a positive regulator) in hypoxic segments. Segments obtained from normoxic rats had detectable basal Ser1177-phosphorylated eNOS (required for efficient nitric oxide production), which increased with carbachol stimulation. No phosphorylated eNOS was found in hypoxic arteries (35).

Statins have been shown to affect all three of these posttranslational regulators of eNOS activity; they decrease caveolin abundance (13), enhance association of eNOS with HSP-90 (6), and increase Ser1177-phosphorylation of eNOS (27). If simvastatin increased eNOS activity in the hypoxia/drug-treated group, then increased NO production and negative feedback may be an additional mechanism to explain the reduced expression of eNOS in the hypoxia/simvastatin-treated rats (16). Additional studies are required to characterize the effects of simvastatin on lung NOS activity in this model.

One potentially important confounder in our study was the unexpected reduction in hematocrit in the simvastatin-hypoxia group, because this effect could partially explain the lower pulmonary artery pressure and right ventricular hypertrophy. Polycythemia and the consequent increase in blood viscosity is an important determinant of the increased pulmonary vascular resistance in response to chronic hypoxia. In chronically hypoxic rats phlebotomized to normocytemia after hypoxia exposure, pulmonary artery pressure and pulmonary vascular resistance were reduced compared with control hypoxic animals but remained higher than normoxic controls (14). Whereas polycythemia clearly contributes to the pulmonary hypertension of chronic hypoxia, pulmonary vascular remodeling is not altered by reductions in hematocrit. Janssens et al. (24) repeatedly phlebotomized guinea pigs during chronic hypoxic exposure to maintain a hematocrit of 46% compared with 57% in unbled hypoxic animals. Pulmonary artery pressure and right ventricular hypertrophy were reduced but without alteration in medial thickness or small vessel neomuscularization. Similar findings were reported in chronically hypoxic mice prevented from developing polycythemia by repeated bleeding (36). Thus chronic hypoxia, and not polycythemia, is the primary stimulus that leads to structural changes within the pulmonary vasculature.

The basis for our observed reduction in hematocrit in the hypoxia/simvastatin-treated group is not clear. No change in hematocrit was observed in the normoxia/simvastatin-treated rats compared with normoxic controls. There are no reports of hematopoietic toxicity with simvastatin treatment or a direct effect on erythropoietin expression. A few of the animals in the simvastatin-treated groups did develop small abdominal wall hematomas at the site of the intraperitoneal injections, which were not seen with injection of vehicle. These drugs have recognized antithrombotic properties (42), which may have promoted bleeding and consequently prevented the expected polycythemia associated with hypoxia. The simvastatin-treated rats gained less weight than their respective control groups, raising the possibility that nutritional factors may have impaired erythropoiesis. Hepatocellular injury is a recognized toxicity of statins (15) but is not generally observed at the dose employed in this study. We examined the liver of one of the hypoxia/simvastatin-treated animals and failed to observe any histological abnormalities.

Simvastatin may have somehow interfered with the polycythemic response to hypoxia. This response is mediated through activation of hypoxia-inducible factor-1α (HIF-1α), which subsequently induces erythropoietin expression (25). Generation of reactive oxygen species during hypoxia stabilizes HIF-1α protein, allowing it to accumulate (8). The antioxidant effect of statins would be expected to suppress reactive oxygen species generation during hypoxia and could thereby reduce HIF-1α accumulation. In addition, Rac and Ras, two small G proteins that require isoprenylation for their activity, are involved in the erythropoietin signal transduction pathway in hematopoietic cells (2). Other agents that have been shown to attenuate chronic hypoxic pulmonary hypertension in rats have also suppressed the polycythemic response (18, 34).

In summary, we have shown that treatment with simvastatin significantly attenuates pulmonary hypertension, polycythemia, and pulmonary vascular remodeling in chronically hypoxic rats. Enhancement of lung eNOS expression does not appear to be involved in mediating this effect. Further studies are required to confirm our findings and delineate the mechanism(s). Statins may prove to be a useful adjunct to currently available therapies for pulmonary hypertension.

REFERENCES

embracing the need for new insights in the field of pulmonary hypertension.


