Simvastatin Attenuates Smooth Muscle Neointimal Proliferation and Pulmonary Hypertension in Rats

Toshihiko Nishimura, John L. Faul, Gerald J. Berry, Laszlo T. Vaszar, Daoming Qiu, Ronald G. Pearl, and Peter N. Kao

Division of Pulmonary and Critical Care Medicine, and Departments of Pathology and Anesthesiology, Stanford University Medical Center, Stanford, California

Hypertensive pulmonary vascular disease is characterized by abnormal proliferation of vascular endothelial and smooth muscle cells, leading to occlusion of pulmonary arterioles, pulmonary hypertension, right ventricular failure, and death. Compounds with antiproliferative effects on vascular endothelial and smooth muscle cells, such as 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, may prevent the development of experimental hypertensive pulmonary vascular disease. Pneumonectomized rats injected with monocrotaline at 7 days develop severe hypertensive pulmonary vascular disease with neointimal formation. Rats were randomized to receive either vehicle or treatment with the HMG-CoA reductase inhibitor simvastatin (2 mg/kg per day). By Day 35, rats that received vehicle had higher mean pulmonary arterial pressures (53 ± 2 mm Hg) and right ventricular hypertrophy (right ventricle/[left ventricle plus septum] [RV/LV+S] = 0.78 ± 0.09) than rats in Group PMS_{5-35} that received simvastatin from Day 5 to 35 (mean pulmonary arterial pressure = 27 ± 3 mm Hg, RV/LV+S = 0.34 ± 0.08; $p \leq 0.001$). Pulmonary vascular remodeling with neointimal formation consisting of vascular smooth muscle cells was more severe in vehicle-treated rats (vascular occlusion score, 1.98 ± 0.02) than in Group PMS_{5-35} (vascular occlusion score, 0.59 ± 0.46; $p < 0.001$). In addition, lung endothelial nitric oxide synthase gene expression was decreased in vehicle-treated animals but was restored toward normal levels in simvastatin-treated animals. Simvastatin attenuates monocrotaline-induced pulmonary vascular remodeling with neointimal formation, pulmonary arterial hypertension, and right ventricular hypertrophy in rats.

Keywords: cholesterol; endothelium; hypertension, pulmonary; nitric oxide synthase

Hypertensive pulmonary vascular disease presents with insidious dyspnea on exertion and is characterized pathophysiologically by pulmonary arterial hypertension and progressive right ventricular failure. The pathological changes of hypertensive pulmonary vascular disease involve abnormal proliferation of vascular endothelial and smooth muscle cells, resulting in occlusion of the lumen of small pulmonary arteries (1–3). Mutations in the bone morphogenetic protein receptor Type II (BMPR2) have been demonstrated in patients with familial and sporadic primary pulmonary hypertension (PPH) (4, 5). Defective BMPR2 signaling may contribute to the intimal proliferation and vascular obliteration of small pulmonary arterioles in PPH (6). The best currently established treatments for PPH involve administration of prostanoids (7), which dilate reactive blood vessels and suppress the growth of smooth muscle cells (8). Additional antiproliferative strategies for treatment of vascular occlusive diseases are warranted (9).

Our group has previously demonstrated that antiproliferative drugs with no acute vasodilator properties effectively attenuate vascular remodeling with neointimal formation, pulmonary arterial hypertension, and right ventricular hypertrophy in pneumonectomized, monocrotaline-injected rats (10, 11). The combination of pneumonectomy with monocrotaline injection results in more severe pulmonary arterial hypertension (mean pulmonary arterial blood pressure [Ppa] = 45 mm Hg) (10–12) than monocrotaline alone (Ppa = 32 mm Hg) (13–15). Importantly, only the combination of pneumonectomy with monocrotaline injection produces neointimal formation and vascular obliteration of small pulmonary arterioles that reproduces many of the pathological features of human PPH (2, 9).

It is increasingly recognized that treatment with 3-hydroxymethyl-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, statins, produces improvements in cardiovascular outcomes that are incompletely explained by reductions in cholesterol (16, 17). Statins exert direct antiinflammatory and antiproliferative effects on the components of vascular wall (18, 19). These mechanisms involve inhibition of isoprenylation of Rho and Rac family GTPases that couple tyrosine kinase growth factor membrane receptors to the intracellular mitogen-activated protein/extracellular signal-regulated kinase inflammatory/proliferative signaling pathways (18, 19). In pulmonary vein endothelial cells, simvastatin blocks activation of the calcium-dependent focal-adhesion tyrosine kinase, Pyk2, necessary for angiotensin II receptor-mediated inflammatory signaling (20). Simvastatin inhibited human aortic vascular smooth muscle cell proliferation through induction of the cell cycle inhibitor, $p27^{Kip1}$ (18). Statins also improve endothelium-dependent relaxation through mechanisms that involve stabilization of endothelial nitric oxide synthase (eNOS) mRNA during hypoxia (21), and enhancement of Akt/protein kinase B phosphorylation of eNOS, leading to increased production of nitric oxide (22). Statins suppressed neointimal formation and vascular narrowing after balloon injury of carotid arteries in normocholesterolemic rabbits (23).

On the basis of the benefits demonstrated by statins in decreasing neointimal vascular occlusion and improving blood flow in the systemic arterial circulation (17, 24–27), we hypothesized that statins might show benefits in experimental hypertensive pulmonary vascular disease. Here, we characterized the effects of simvastatin on development of neointimal formation, pulmonary arterial hypertension, and right ventricular hypertrophy in pneumonectomized, monocrotaline-treated rats.

(*Received in original form March 29, 2002; accepted in final form August 6, 2002*)

Supported by a gift from the Donald E. and Delia B. Baxter Foundation, and by NIH grants AI39624 and HL62588 to P.N.K.

Correspondence and requests for reprints should be addressed to Peter N. Kao, M.D., Ph.D., Division of Pulmonary and Critical Care Medicine, Stanford University Medical Center, Stanford, CA 94305-5236. E-mail: peterkao@stanford.edu

Am J Respir Crit Care Med Vol 166. pp 1403–1408, 2002
Originally Published in Press as DOI: 10.1164/rccm.200203-268OC on August 15, 2002
Internet address: www.atsjournals.org

METHODS

Study Sample

Thirty pathogen-free, 13-week-old, male Sprague-Dawley rats (body weight, 350–400 g) were studied.

Left Pneumonectomy

On Day 0, rats were intubated, anesthetized with halothane (0.5%), and then underwent left pneumonectomy via left thoracotomy, as previously described (10, 11).

Monocrotaline Administration

On Day 7, rats were injected subcutaneously in the right hind limb with monocrotaline (60 mg/kg; Sigma, St. Louis, MO).

Treatment Groups

Rats were randomized to receive simvastatin or vehicle by daily gavage. Five groups were studied: Group $PMS_{5–35}$ (whole treatment group) received simvastatin (2 mg/kg per day) from Day 5 to Day 35 (n = 6), Group $PMS_{5–14}$ (early treatment group) received simvastatin (2 mg/kg per day) from Day 5 to Day 14 (n = 6), and Group $PMS_{15–35}$ (late treatment group) received simvastatin (2 mg/kg per day) from Day 15 to Day 35 (n = 6). Group PMV (vehicle group) received a vehicle (placebo) solution from Day 5 to Day 35 (n = 6). An additional six rats were studied as a normal (control) group. These rats did not undergo pneumonectomy, nor did they receive monocrotaline or simvastatin.

All animals received humane care and the study was approved by the Stanford University (Stanford, CA) Panel on Laboratory Animal Care.

Hemodynamic Studies and Tissue Preparation

On Day 35, rats were anesthetized with halothane. Mean arterial, mean pulmonary arterial, and right ventricular systolic blood pressures were measured as previously described (10, 11). After exsanguination, the right lung, right ventricle, and left ventricle plus septum were collected for histology (10, 11). The severity of neointimal formation was scored on lung sections stained with elastin–van Gieson: the absence of neointimal formation equals 0, the presence of neointimal proliferation causing less than 50% lumenal narrowing equals 1, and lumenal narrowing greater than 50% equals 2 (10, 11). Immunohistochemistry was performed on formalin-fixed, paraffin-embedded lung sections, using antibodies to α-smooth muscle actin (1:30 dilution, linked to alkaline phosphatase; Dako, Carpinteria, CA) and CD31 (1:40 dilution, detected by the avidin–biotin–peroxidase method; Dako). Samples of right lung were immediately placed in 2.5% paraformaldehyde and routinely processed for transmission electron microscopy

Endothelial Nitric Oxide Synthase Expression Analysis

Reverse transcription-polymerase chain reaction (RT-PCR) was used to amplify portions of the rat endothelial nitric oxide synthase gene (eNOS; GenBank accession number U02534) and actin B (GenBank accession number V01217) from rat lung. Primer sequences for eNOS were as follows: 5′-ctgggctccagagcataccc-3′ and 5′-gcagccttggcatcttctcc-3′. Five micrograms of total lung RNA was reverse transcribed and amplified by PCR as described (28). Endothelial NOS protein (60 μg) was analyzed by Western immunoblotting (29), using anti-eNOS primary antibody (sc-8311; Santa Cruz Biotechnology, Santa Cruz, CA).

Statistical Analysis

Data are presented as means ± standard deviation. Groups PMV, $PMS_{5–35}$, $PMS_{5–14}$, and $PMS_{15–35}$ were analyzed by two-way analysis of variance (ANOVA) with multiple comparisons. This analysis allows us to detect whether early or late treatment with simvastatin produces any significant effects compared with vehicle and whether there is any interaction between early and late simvastatin treatments. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Twenty-four rats received monocrotaline (60 mg/kg, subcutaneous), 7 days after pneumonectomy. All rats in the four treatment groups survived for the duration of the study and underwent hemodynamic measurements followed by sacrifice on postoperative Day 35. The normal group consisted of six normal, healthy rats that were included as a reference control group for changes in body weight, hemodynamic parameters, organ weights, and histopathology.

Body Weights

During the 35-day study period, rats in the normal group increased in body weight by 18%. In contrast, Group PMV rats, which developed severe hypertensive pulmonary vascular disease after pneumonectomy, monocrotaline injection, and vehicle treatment, decreased in body weight by 15%. Rats treated with simvastatin after pneumonectomy and monocrotaline injection preserved their body weights (data not shown).

Hemodynamic Parameters

Hemodynamic measurements of rats in the normal group demonstrated a mean arterial blood pressure of 126 ± 9 mm Hg, a mean pulmonary arterial blood pressure ($\overline{Ppa}$) of 17 ± 1 mm Hg, typical of healthy adult rats (30, 31).

Arterial blood pressure. Pneumonectomized, monocrotaline-injected rats treated with simvastatin showed no significant difference in systemic arterial blood pressure compared with normal rats.

Pulmonary arterial pressure and right ventricular systolic blood pressure. Pneumonectomized, monocrotaline-injected rats treated with simvastatin had lower Ppa and mean right ventricular systolic pressure ($\overline{Prv,s}$) values than rats that received vehicle (Figures 1A and 1B). Rats in Group $PMS_{5–35}$ (whole treatment) had the lowest ($\overline{Ppa}$) (27 ± 3 mm Hg) and $\overline{Prv,s}$ (37 ± 4 mm Hg) values. Rats in Group $PMS_{15–35}$ (late treatment) had lower Ppa (38 ± 3 mm Hg) and $\overline{Prv,s}$ (54 ± 10 mm Hg) values than those in Group $PMS_{5–14}$ (early treatment) (($\overline{Ppa}$) = 44 ± 4 mm Hg; $\overline{Prv,s}$ = 66 ± 12 mm Hg). Of the four groups, rats in Group PMV (received vehicle) had the highest ($\overline{Ppa}$) (53 ± 2 mm Hg) and $\overline{Prv,s}$ (81 ± 6 mm Hg) values. Two-way ANOVA and multiple comparison revealed that significant effects of simvastatin treatment on Ppa occurred independently in the early simvastatin treatment group (Group $PMS_{5–14}$, $p < 0.001$) and in the late simvastatin treatment group (Group $PMS_{15–35}$, $p < 0.001$).

Organ Weights

Right ventricular hypertrophy. The development of chronic pulmonary arterial hypertension results in a compensatory hypertrophy of the right ventricle (increased ratio of right ventricle to left ventricle plus septum; RV/LV+S). The RV/LV+S ratio in normal rats was 0.25 ± 0.03. Rats treated with simvastatin had lower RV/LV+S values than rats that received vehicle (Group PMV, RV/LV+S = 0.78 ± 0.09) (Figure 2). The mean RV/LV+S ratios for the simvastatin-treated animals were as follows: Group $PMS_{5–35}$, 0.34 ± 0.07; Group $PMS_{5–14}$, 0.66 ± 0.11; and Group $PMS_{15–35}$, 0.60 ± 0.15 (Figure 2). Two-way ANOVA and multiple comparison revealed that the main effect occurred in the late simvastatin treatment group (Group $PMS_{15–35}$, $p < 0.01$).

Histopathology

Representative small pulmonary arteries from normal rats and pneumonectomized, monocrotaline-injected rats treated with vehicle or simvastatin are shown, stained for elastin to reveal the inner elastic lamina (Figures 3A–3E). A quantitative analysis of lumenal obstruction in 25 consecutive small pulmonary arter-

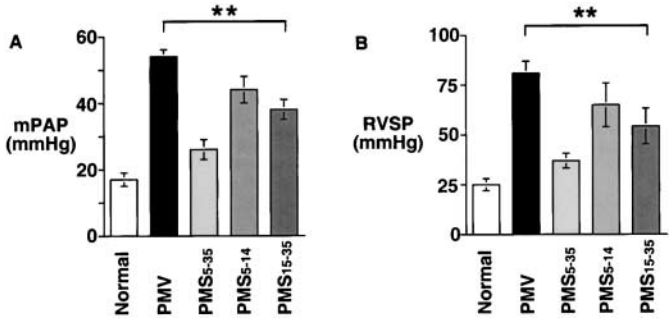


Figure 1. Simvastatin prevents the development of pulmonary arterial hypertension in pneumonectomized, monocrotaline-treated rats. Shown are measurements of (*A*) mean pulmonary artery pressure (mean (Ppa), or mPAP) and (*B*) right ventricular systolic pressure (Prv,s, or RVSP) in normal (control) group, Group PMV, Group $PMS_{5–35}$, Group $PMS_{5–14}$, and Group $PMS_{15–35}$ rats (each group, n = 6).

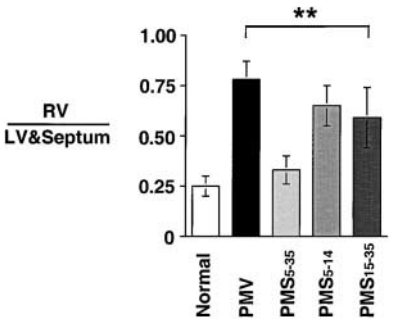
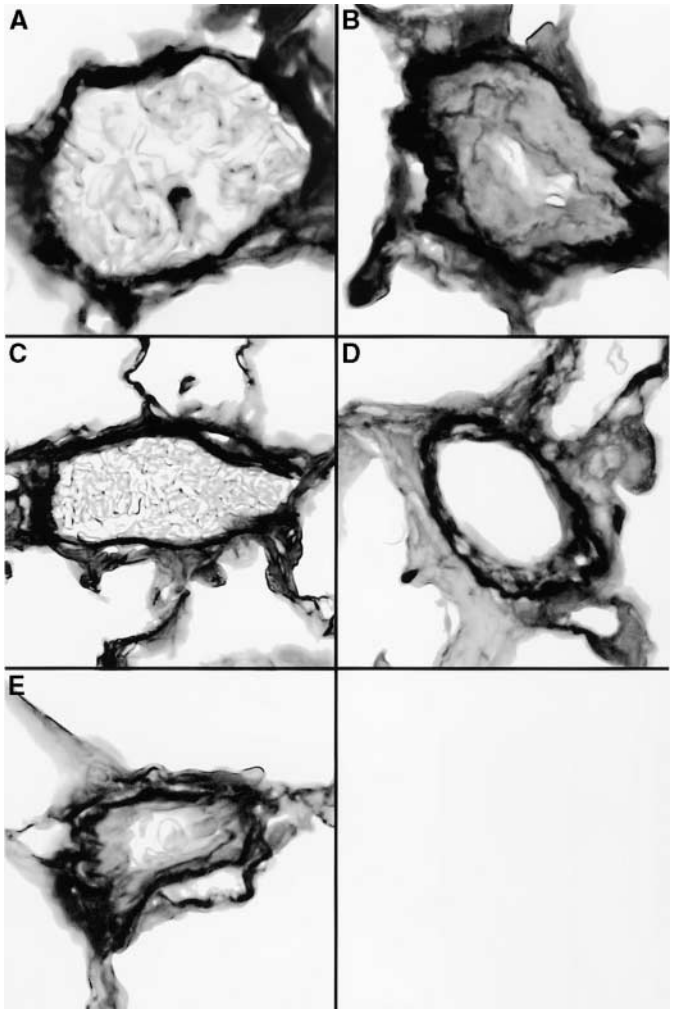


Figure 2. Simvastatin prevents the development of right ventricular hypertrophy in pneumonectomized, monocrotaline-treated rats. Shown are ratios of right ventricular mass to left ventricle plus septal mass (RV/LV+S) in normal (control), Group PMV, Group $PMS_{5–35}$, Group $PMS_{5–14}$, and Group $PMS_{15–35}$ rats (each group, n = 6).

ies from each rat in the normal group and Groups PMV, $PMS_{5–35}$, $PMS_{5–14}$, and $PMS_{15–35}$ was performed. The distribution of the vascular lesions, and an average vascular occlusion score (VOS) between 0 and 2, are presented (Figure 3F). Rats in Group PMV developed severe pulmonary vascular remodeling with a VOS of 1.98 ± 0.02 (Figure 3B versus Figure 3A) (10, 11).

Rats treated with simvastatin showed significant decreases in VOS compared with vehicle-treated rats. Rats in Group $PMS_{5–35}$ showed a VOS of 0.59 ± 0.46, rats in Group $PMS_{5–14}$ showed a VOS of 0.78 ± 0.40, and rats in Group $PMS_{15–35}$ showed a VOS of 0.93 ± 0.38. Two-way ANOVA and multiple comparison revealed that significant effects of simvastatin treatment on VOS occurred in both the early simvastatin treatment group (Group $PMS_{5–14}$, $p < 0.001$) and in the late simvastatin treatment group (Group $PMS_{15–35}$, $p < 0.001$). The improvements in VOS achieved in all groups treated with simvastatin substantially exceeded the best improvements achieved with triptolide (VOS = 1.27 ± 0.61)



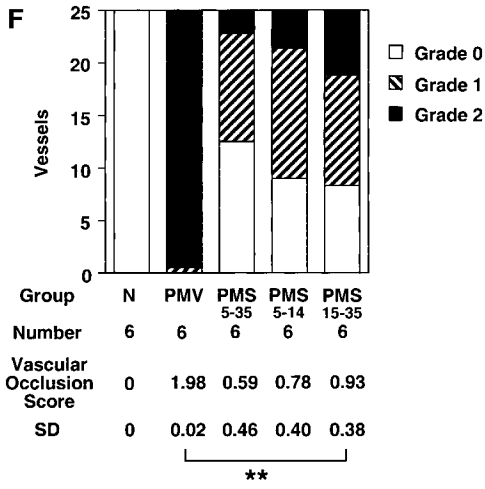


Figure 3. Simvastatin attenuates pulmonary artery neointimal formation. The vascular occlusion score is the average score for 25 consecutive intra-acinar pulmonary arteries. Vessels were assessed for neointimal proliferative lesions and score: Grade 0 for no evidence of neointimal formation, Grade 1 for less than 50% lumenal occlusion, and Grade 2 for greater than 50% lumenal occlusion. (*A*) Normal rat intra-acinar artery (Grade 0) (elastin–van Gieson stain; original magnification, ×600); (*B*) Grade 2 neointimal lesion (> 50% narrowing) in pneumonectomized rats, 4 weeks after receiving monocrotaline (60 mg/kg, subcutaneous). (*C*) Grade 1 lesion showing minimal intimal thickening in Group $PMS_{5–35}$ (whole treatment group). (*D*) Grade 1 lesion in Group $PMS_{5–14}$ (early treatment group). (*E*) Grade 1 lesion in Group $PMS_{15–35}$ (late treatment group). (*F*) Grades of vascular occlusion in right lung of Group N (normal), Group PMV, Group $PMS_{5–35}$ (whole treatment group), Group $PMS_{5–14}$ (early treatment group), and Group $PMS_{15–35}$ (late treatment group) rats (each group, n = 6).

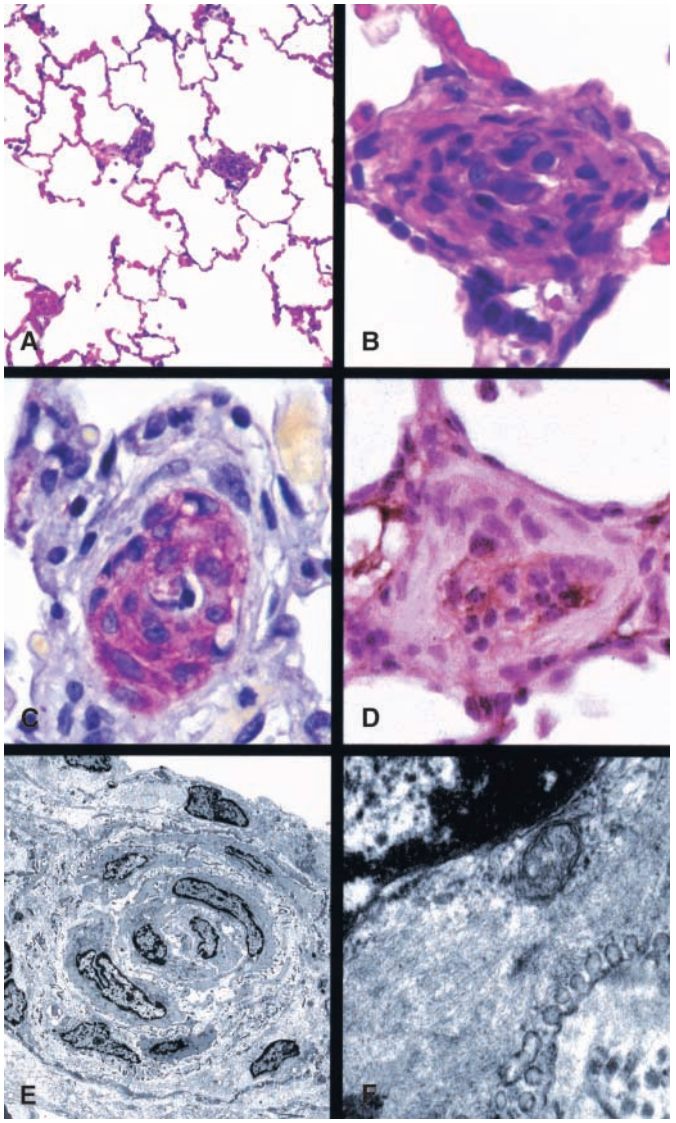


Figure 4. Smooth muscle cells are present in intimal lesions of small pulmonary arteries. (*A*) Occlusion of small intra-acinar pulmonary arteries on Day 35 in Group PMV rats with severe hypertensive pulmonary vascular disease (hematoxylin and eosin stain; original magnification, ×200). (*B*) Grade 2 neointimal lesion (hematoxylin and eosin stain; original magnification, ×600). (*C*) Most cells in Grade 2 lesion stain positive (*red*) for α-smooth muscle actin. (*D*) CD31-positive staining (*brown*) is limited to a layer of cells lining the vascular lumen of a Grade 2 lesion. (*E* and *F*) Electron microscopy of Grade 2 lesion demonstrates smooth muscle differentiation. Original magnification: (*E*) ×12,000; (*F*) ×80,000.

(10) or with 40-*O*-(2-hydroxyethyl)-rapamycin (SDZ-RAD; VOS = 1.57 ± 0.1) (11).

The cellular phenotype of the pulmonary microvascular neointimal occlusive lesions was characterized. Hematoxylin- and eosin-stained lung sections from Group PMV rats demonstrate extensive occlusion of small pulmonary arterioles, with preservation of alveolar structures (Figures 4A and 4B). These consisted of concentric laminar proliferations of spindled cells. Plexiform lesions in muscular pulmonary arteries were not observed. Immunohistochemical staining reveals the presence of α-smooth muscle actin staining in many of the cells forming the neointima (Figure 4C). A marker of endothelial cells is CD31; an anti-

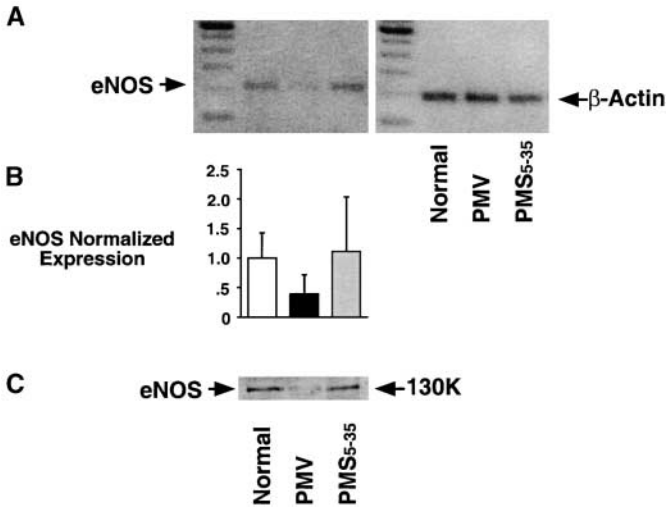


Figure 5. Decrease in endothelial nitric oxide synthase (eNOS) gene expression during the development of hypertensive pulmonary vascular disease and its restoration by simvastatin. (*A*) Reverse transcription-PCR analysis of eNOS and β-actin gene expression in representative rat lungs from Group N, Group PMV, and Group $PMS_{5–35}$ (whole treatment group). (*B*) Mean and standard deviation of eNOS mRNA expression normalized to β-actin is shown (each group, n = 4). (*C*) Western immunoblot of eNOS protein expression in representative rat lung protein extracts from normal, Group PMV, and Group $PMS_{5–35}$ (whole treatment group) rats.

CD31 monoclonal antibody is shown in Figure 4D to stain only cells lining the vascular lumen. We used electron microscopy to further characterize the neointimal lesions (Figures 4E and 4F) and show the presence of alternating plaques and caveolae at the surface. No fibronectin fibrils or fibronexus junctions, which are features of myofibroblastic differentiation, were seen. Taken together, the presence of α-smooth muscle actin and these ultrastructural findings identifies these cells in the neointima to be smooth muscle cells (32).

Cholesterol

Rats treated with simvastatin showed no significant differences in serum cholesterol on Day 35 compared with normal rats.

Endothelial Nitric Oxide Synthase Gene Expression

Changes in lung eNOS mRNA expression were assessed semiquantitatively by RT-PCR (Figure 5A). The expression of eNOS mRNA, normalized to β-actin expression, was significantly lower in diseased rats (Group PMV) than in normal rats (Figure 5B). Simvastatin treatment increased expression of eNOS in pneumonectomized, monocrotaline-injected rats, although this trend did not achieve statistical significance.

The lung expression of eNOS protein was assessed qualitatively by Western immunoblotting (Figure 5C). An eNOS antiserum-immunoreactive band is detected at 130 kDa (Figure 5C). Compared with normal rats, diseased rats in Group PMV showed decreased expression of eNOS protein, and simvastatin treatment restored eNOS expression toward the level in normal rats (Figure 5C).

DISCUSSION

Pulmonary arterial hypertension involves histopathological changes of medial hypertrophy, intimal proliferation, and occlu-

sion of microvascular pulmonary arteries and, in severe cases, plexiform arteriopathy (2). The nature and origin of the cells present in the obstructing intimal lesions represent important questions for further understanding of the pathogenesis of hypertensive pulmonary vascular disease. In a comprehensive analysis of 53 lungs from patients with moderate to severe pulmonary arterial hypertension (as a consequence of primary pulmonary hypertension, Eisenmenger's syndrome, and chronic thromboembolic disease), Yi and coworkers (2) demonstrated that most cells within intimal lesions stained positive for α-smooth muscle actin, and negative for endothelial markers, which the authors proposed as support for a myofibroblastic phenotype. Only a single layer of cells that lined the vascular lumen stained positively for endothelial markers, CD31 and Factor VIII (2). The results of Yi and coworkers (2) differ from the earlier description by Tuder and coworkers (33), who used Factor VIII immunoreactivity to propose an endothelial origin of the cells present in plexiform lesions of patients with PPH. These differing histopathological results might be reconciled in light of demonstrations that transforming growth factor-β can induce a small percentage of endothelial cells to transdifferentiate into smooth muscle-like cells (34–36). The transdifferentiating endothelial cells simultaneously stained positive with antibodies against Factor VIII-related antigen and α-smooth muscle actin (34, 36).

Our model of hypertensive pulmonary vascular disease in pneumonectomized, monocrotaline-injected rats reproduces the neointimal proliferation and vascular occlusion by smooth muscle cells that occurs in human pulmonary arterial hypertension. When rats are injected with the *Crotalaria* toxin, monocrotaline, there is hepatic metabolism of the monocrotaline into monocrotaline pyrrole, which then produces first-pass injury to the pulmonary artery endothelial cells (31, 37). Endothelial injury by monocrotaline pyrrole leads to diminished barrier function, allowing extravascular leakage of proteases such as serum elastase and thrombin that act on extracellular matrix components and vascular cells to trigger an inflammatory response (15, 37, 38). Endothelial injury followed by an inflammatory response and eventual neointimal vascular occlusion by smooth muscle cells represents a valid and important model system for understanding the development and progression of small-vessel obliterative, hypertensive pulmonary vascular disease in humans.

We demonstrate that simvastatin is effective at attenuating vascular remodeling with neointimal formation, pulmonary arterial hypertension, and right ventricular hypertrophy in pneumonectomized, monocrotaline-injected rats. We previously characterized the effects of two immunosuppressants with antiproliferative effects, triptolide (10) and rapamycin (11), using this experimental model. What mechanisms are involved in the efficacy of simvastatin in attenuating hypertensive pulmonary vascular disease?

Although less immunosuppressive than either triptolide or rapamycin, statins do modulate the immune response by repressing MHC Class II-mediated T cell activation (39). Immunosuppressive and antiinflammatory properties of statins may contribute to the improved outcome of cardiac transplant patients under treatment with pravastatin (40) and to the improved survival of patients with atherosclerosis, recognized to be a chronic inflammatory disease (16, 17, 41). Our rat model of hypertensive pulmonary vascular disease involves an early inflammatory response after monocrotaline-induced injury of pulmonary endothelial cells (37), followed subsequently by a vascular proliferative response that produces pulmonary hypertension. The efficacy of simvastatin in the whole and early treatment groups of our disease model may involve antiinflammatory effects.

Chronic treatment with statins improves coronary (24) and cerebral (25, 26) blood flow, through mechanisms that involve induction of eNOS and improvements in endothelium-dependent vasodilation (27). We infer that simvastatin improved pulmonary vascular blood flow from the decreases in mean pulmonary arterial pressures, decreases in right ventricular hypertrophy, and decreases in vascular occlusion scores on histology. Continuous treatment with simvastatin resulted in a Ppa of 27 mm Hg compared with 53 and 17 mm Hg in vehicle-treated and normal rats, respectively. This decrease achieved with simvastatin appeared greater than that with triptolide (21 versus 42 mm Hg) (10) or with a rapamycin derivative, SDZ-RAD (25 versus 41 mm Hg) (11). Simvastatin treatment appeared to produce greater protection against the development of right ventricular hypertrophy (RV/LV+S, 0.34 versus 0.78) than did triptolide (RV/LV+S, 0.39 versus 0.69) or SDZ-RAD (RV/LV+S, 0.42 versus 0.55). Simvastatin treatment appeared to produce a greater decrease in neointimal formation (VOS = 0.59) compared with triptolide-treated (VOS = 1.27), RAD-treated (VOS = 1.57), and control rats with hypertensive pulmonary vascular disease (VOS = 1.98). Simvastatin attenuated hypertensive pulmonary vascular disease significantly in both the early and late treatment groups. The efficacy of simvastatin in the late treatment group suggests its ability to rescue animals from established hypertensive pulmonary vascular disease.

In other systems, simvastatin has been demonstrated to inhibit vascular smooth muscle cell proliferation *in vitro* (18) and *in vivo* (19) and to suppress neointimal proliferation (19, 23). In this model of hypertensive pulmonary vascular disease, we demonstrate that simvastatin attenuates neointimal proliferation and decreases pulmonary arterial hypertension through mechanisms that likely involve inhibition of pulmonary vascular smooth muscle cell proliferation *in vivo*.

Human patients with pulmonary hypertension show reduced expression of eNOS (42). Our demonstration of reduced expression of eNOS in diseased rats further supports the similarities between our experimental model and human hypertensive pulmonary vascular disease. Simvastatin induces expression of eNOS *in vitro* (21). We show that simvastatin augmented lung expression of eNOS mRNA and protein in diseased rats. In other systems, simvastatin augmentation of eNOS expression and stimulation of eNOS phosphorylation by protein kinase Akt were associated with enhanced production of the vasodilator nitric oxide (22).

The efficacy of simvastatin in attenuating neointimal vascular occlusion, pulmonary hypertension, and right ventricular hypertrophy in this model suggests again the utility of antiproliferative treatment strategies for hypertensive pulmonary vascular disease (10, 11). We propose that simvastatin attenuation of hypertensive pulmonary vascular disease involves a combination of (*1*) antiproliferative effects on pulmonary vascular smooth muscle cells, (*2*) antiinflammatory effects that include decreases in endothelial MHC Class II expression, and (*3*) induction of endothelial cell eNOS expression and restoration of endothelium-dependent pulmonary vasodilation.

An important question raised by this study is whether simvastatin treatment can reverse established neointimal vascular occlusion and pulmonary hypertension. We have developed a new model of chronic pulmonary arterial hypertension in rats for evaluation of rescue therapies such as simvastatin (T. Nishimura, unpublished data). We suggest that simvastatin should be evaluated both for prevention of pulmonary hypertension in genetically susceptible patients (43) as well as for treatment of patients with established pulmonary hypertension.

Acknowledgment: The authors thank Professor Yoshinori Fujii, Department of Statistics, Stanford University, for statistical advice and Gail V. Benson for technical assistance.

References

1. Fishman AP, Fishman MC, Freeman BA, Gimbrone MA, Rabinovitch M, Robinson D, Gail DB. Mechanisms of proliferative and obliterative vascular diseases: insights from the pulmonary and systemic circulations. NHLBI Workshop Summary. *Am J Respir Crit Care Med* 1998; 158:670–674.
2. Yi ES, Kim H, Ahn H, Strother J, Morris T, Masliah E, Hansen LA, Park K, Friedman PJ. Distribution of obstructive intimal lesions and their cellular phenotypes in chronic pulmonary hypertension: a morphometric and immunohistochemical study. *Am J Respir Crit Care Med* 2000;162:1577–1586.
3. Strauss BH, Rabinovitch M. Adventitial fibroblasts: defining a role in vessel wall remodeling. *Am J Respir Cell Mol Biol* 2000;22:1–3.
4. Deng Z, Morse JH, Slager SL, Cuervo N, Moore KJ, Venetos G, Kalachikov S, Cayanis E, Fischer SG, Barst RJ, *et al.* Familial primary pulmonary hypertension (gene PPH1) is caused by mutations in the bone morphogenetic protein receptor-II gene. *Am J Hum Genet* 2000;67: 737–744.
5. Lane KB, Machado RD, Pauciulo MW, Thomson JR, Phillips JA III, Loyd JE, Nichols WC, Trembath RC. Heterozygous germline mutations in BMPR2, encoding a TGF-β receptor, cause familial primary pulmonary hypertension. International PPH Consortium. *Nat Genet* 2000;26:81–84.
6. Atkinson C, Stewart S, Upton PD, Machado R, Thomson JR, Trembath RC, Morrell NW. Primary pulmonary hypertension is associated with reduced pulmonary vascular expression of Type II bone morphogenetic protein receptor. *Circulation* 2002;105:1672–1678.
7. Barst RJ, Rubin LJ, Long WA, McGoon MD, Rich S, Badesch DB, Groves BM, Tapson VF, Bourge RC, Brundage BH, *et al.* A comparison of continuous intravenous epoprostenol (prostacyclin) with conventional therapy for primary pulmonary hypertension. Primary Pulmonary Hypertension Study Group. *N Engl J Med* 1996;334:296–302.
8. Clapp LH, Finney P, Turcato S, Tran S, Rubin LJ, Tinker A. Differential effects of stable prostacyclin analogs on smooth muscle proliferation and cyclic AMP generation in human pulmonary artery. *Am J Respir Cell Mol Biol* 2002;26:194–201.
9. Newman JH, Lane KB. Hypertensive pulmonary vascular disease: dawn of the age of prevention? *Am J Respir Crit Care Med* 2000;162:2020–2021.
10. Faul JL, Nishimura T, Berry GJ, Benson GV, Pearl RG, Kao PN. Triptolide attenuates pulmonary arterial hypertension and neointimal formation in rats. *Am J Respir Crit Care Med* 2000;162:2252–2258.
11. Nishimura T, Faul JL, Berry GJ, Veve I, Kao PN, Pearl RG. 40-*O*-(2-Hydroxyethyl)-rapamycin attenuates pulmonary arterial hypertension and neointimal formation in rats. *Am J Respir Crit Care Med* 2001; 163:498–502.
12. Okada K, Tanaka Y, Bernstein M, Zhang W, Patterson GA, Botney MD. Pulmonary hemodynamics modify the rat pulmonary artery response to injury: a neointimal model of pulmonary hypertension. *Am J Pathol* 1997;151:1019–1025.
13. Rosenberg HC, Rabinovitch M. Endothelial injury and vascular reactivity in monocrotaline pulmonary hypertension. *Am J Physiol* 1988;255: H1484–H1491.
14. Mathew R, Zeballos GA, Tun H, Gewitz MH. Role of nitric oxide and endothelin-1 in monocrotaline-induced pulmonary hypertension in rats. *Cardiovasc Res* 1995;30:739–746.
15. van Suylen RJ, Smits JF, Daemen MJ. Pulmonary artery remodeling differs in hypoxia- and monocrotaline-induced pulmonary hypertension. *Am J Respir Crit Care Med* 1998;157:1423–1428.
16. Koh KK. Effects of statins on vascular wall: vasomotor function, inflammation, and plaque stability. *Cardiovasc Res* 2000;47:648–657.
17. Maron DJ, Fazio S, Linton MF. Current perspectives on statins. *Circulation* 2000;101:207–213.
18. Laufs U, Marra D, Node K, Liao JK. 3-Hydroxy-3-methylglutaryl-CoA reductase inhibitors attenuate vascular smooth muscle proliferation by preventing rho GTPase-induced down-regulation of $p27^{Kip1}$. *J Biol Chem* 1999;274:21926–21931.
19. Indolfi C, Cioppa A, Stabile E, Di Lorenzo E, Esposito G, Pisani A, Leccia A, Cavuto L, Stingone AM, Chieffo A, *et al.* Effects of hydroxymethylglutaryl coenzyme A reductase inhibitor simvastatin on smooth muscle cell proliferation in vitro and neointimal formation in vivo after vascular injury. *J Am Coll Cardiol* 2000;35:214–221.
20. Satoh K, Ichihara K, Landon EJ, Inagami T, Tang H. 3-Hydroxy-3-methylglutaryl-CoA reductase inhibitors block calcium-dependent tyrosine kinase Pyk2 activation by angiotensin II in vascular endothelial cells: involvement of geranylgeranylation of small G protein Rap1. *J Biol Chem* 2001;276:15761–15767.
21. Laufs U, Fata VL, Liao JK. Inhibition of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase blocks hypoxia-mediated down-regulation of endothelial nitric oxide synthase. *J Biol Chem* 1997;272:31725–31729.
22. Kureishi Y, Luo Z, Shiojima I, Bialik A, Fulton D, Lefer DJ, Sessa WC, Walsh K. The HMG-CoA reductase inhibitor simvastatin activates the protein kinase Akt and promotes angiogenesis in normocholesterolemic animals. *Nat Med* 2000;6:1004–1010.
23. Soma MR, Donetti E, Parolini C, Mazzini G, Ferrari C, Fumagalli R, Paoletti R. HMG CoA reductase inhibitors: in vivo effects on carotid intimal thickening in normocholesterolemic rabbits. *Arterioscler Thromb* 1993;13:571–578.
24. Andrews TC, Raby K, Barry J, Naimi CL, Allred E, Ganz P, Selwyn AP. Effect of cholesterol reduction on myocardial ischemia in patients with coronary disease. *Circulation* 1997;95:324–328.
25. Endres M, Laufs U, Huang Z, Nakamura T, Huang P, Moskowitz MA, Liao JK. Stroke protection by 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors mediated by endothelial nitric oxide synthase. *Proc Natl Acad Sci USA* 1998;95:8880–8885.
26. Amin-Hanjani S, Stagliano NE, Yamada M, Huang PL, Liao JK, Moskowitz MA. Mevastatin, an HMG-CoA reductase inhibitor, reduces stroke damage and upregulates endothelial nitric oxide synthase in mice. *Stroke* 2001;32:980–986.
27. O'Driscoll G, Green D, Taylor RR. Simvastatin, an HMG-coenzyme A reductase inhibitor, improves endothelial function within 1 month. *Circulation* 1997;95:1126–1131.
28. Aoki Y, Qiu D, Uyei A, Kao PN. Human airway epithelial cells express interleukin-2 *in vitro. Am J Physiol Lung Cell Mol Physiol* 1997;272: L272–L284.
29. Zhao G, Vaszar LT, Qiu D, Shi L, Kao PN. Antiinflammatory effects of triptolide in human bronchial epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 2000;279:L958–L966.
30. Kay JM. Effect of intermittent normoxia on chronic hypoxic pulmonary hypertension, right ventricular hypertrophy, and polycythemia in rats. *Am Rev Respir Dis* 1980;121:993–1001.
31. Pan LC, Wilson DW, Segall HJ. Strain differences in the response of Fischer 344 and Sprague-Dawley rats to monocrotaline induced pulmonary vascular disease. *Toxicology* 1993;79:21–35.
32. Eyden B. The myofibroblast: an assessment of controversial issues and a definition useful in diagnosis and research. *Ultrastruct Pathol* 2001; 25:39–50.
33. Tuder RM, Groves B, Badesch DB, Voelkel NF. Exuberant endothelial cell growth and elements of inflammation are present in plexiform lesions of pulmonary hypertension. *Am J Pathol* 1994;144:275–285.
34. Arciniegas E, Sutton AB, Allen TD, Schor AM. Transforming growth factor β1 promotes the differentiation of endothelial cells into smooth muscle-like cells in vitro. *J Cell Sci* 1992;103:521–529.
35. Arciniegas E, Ponce L, Hartt Y, Graterol A, Carlini RG. Intimal thickening involves transdifferentiation of embryonic endothelial cells. *Anat Rec* 2000;258:47–57.
36. Frid MG, Kale VA, Stenmark KR. Mature vascular endothelium can give rise to smooth muscle cells via endothelial–mesenchymal transdifferentiation. *Circ Res* 2002;90:1189–1196.
37. Lame MW, Jones AD, Wilson DW, Dunston SK, Segall HJ. Protein targets of monocrotaline pyrrole in pulmonary artery endothelial cells. *J Biol Chem* 2000;275:29091–29099.
38. Rabinovitch M. EVE and beyond, retro and prospective insights. *Am J Physiol Lung Cell Mol Physiol* 1999;277:L5–L12.
39. Kwak B, Mulhaupt F, Myit S, Mach F. Statins as a newly recognized type of immunomodulator. *Nat Med* 2000;6:1399–1402.
40. Kobashigawa JA, Katznelson S, Laks H, Johnson JA, Yeatman L, Wang XM, Chia D, Terasaki PI, Sabad A, Cogert GA, *et al.* Effect of pravastatin on outcomes after cardiac transplantation. *N Engl J Med* 1995; 333:621–627.
41. Bustos C, Hernandez-Presa MA, Ortego M, Tunon J, Ortega L, Perez F, Diaz C, Hernandez G, Egido J. HMG-CoA reductase inhibition by atorvastatin reduces neointimal inflammation in a rabbit model of atherosclerosis. *J Am Coll Cardiol* 1998;32:2057–2064.
42. Giaid A, Saleh D. Reduced expression of endothelial nitric oxide synthase in the lungs of patients with pulmonary hypertension. *N Engl J Med* 1995;333:214–221.
43. Newman JH, Wheeler L, Lane KB, Loyd E, Gaddipati R, Phillips JA III, Loyd JE. Mutation in the gene for bone morphogenetic protein receptor II as a cause of primary pulmonary hypertension in a large kindred. *N Engl J Med* 2001;345:319–324.