Severe pulmonary arterial hypertension (PAH) in adult patients is characterized by progressive narrowing/occlusion of small pulmonary arteries, which frequently leads to right heart failure and death.1,2 Factors thought to contribute to the formation of pulmonary vascular lesions include sustained vasoconstriction, vascular remodeling, and in situ thrombosis. However, it is now widely believed that fixed obstruction resulting from vascular remodeling is the major cause of the elevated vascular resistance in severe, progressive PAH.3,4 Large clinical studies show that only 13% of adult PAH patients have a significant decrease in pulmonary artery pressure during acute vasodilator testing at the time of diagnosis,5 suggesting a major fixed structural but minor reversible vasoconstrictor component in this group of pulmonary vascular diseases.

Reeves et al proposed in 1986 that over time in PAH, the hypertensive component attributable to vasoconstriction decreases, whereas that attributable to fixed obstruction increases.6 This concept cannot be tested clinically, because it is essentially impossible to obtain serial hemodynamic data and matched lung tissue samples for thorough assessment of the lung vascular morphology. At best, a single lung specimen becomes available at the time of transplant or at autopsy. Even data on serial vasodilator testing are exceedingly rare in patients with severe PAH. Furthermore, surprisingly, matched serial hemodynamic and histological analyses of early and progressive pulmonary hypertensive disease has also apparently not been performed in animal models. Thus, to our knowledge, this study is the first serial examination of the relationship between occlusive pulmonary vascular remodeling and pulmonary vasomotor tone in a rat model of severe, progressive PAH.7 Here we attempt to address the question of whether there are drugs (not currently used to treat patients with severe PAH) that can reduce the pulmonary artery pressure in the setting of extensive pulmonary vascular occlusion.

Recent studies indicate that the small GTPase RhoA, a member of the Rho family of small GTP-binding proteins, and one of its downstream effectors, Rho-kinase (RhoA/Rho kinase signaling), play a major role in the Ca2+ sensitization and sustained constriction of vascular smooth muscle cells induced by G protein–coupled receptor agonists.8–10 Selc-
tive Rho kinase inhibitors, such as Y-27632 and fasudil, effectively reverse the sustained vasoconstriction induced by many agonists, including endothelin-1 (ET-1), thromboxane A2, and serotonin, and are now regarded as a novel class of potent vasodilators with multiple other actions. Rho kinase inhibitors have been found to effectively inhibit acute hypoxic pulmonary vasoconstriction. In addition, we and others have shown that Rho kinase inhibitors acutely reverse the elevated pulmonary arterial pressure in 3 different rat models of chronic pulmonary hypertension, i.e., Denver-raised, fawn-hooded rats and chronic hypoxia–induced and monocrotaline-induced pulmonary hypertension. However, although these models develop pulmonary artery remodeling, including adventitial and medial wall thickening and muscularization of normally nonmuscular distal arteries, they lack the hallmark pulmonary artery lesions of human severe PAH, lumen-obliterating endothelial cell proliferation. Taraseviciene-Stewart et al have described a new rat model where PAH is predictably induced by the combination of vascular endothelial growth factor receptor blockade with SUGEN (SU) 5416 and chronic hypoxic exposure. Interestingly, the PAH is progressive in spite of reexposure of the animals to normoxia. This severe, progressive PAH is associated with precapillary arterial occlusion by proliferating factor VIII–positive endothelial cells, and, notably, the PAH in these animals is also resistant to treatment with drugs conventionally used to treat the human disease. Thus, this model more closely mimics human severe PAH than the chronic hypoxia and monocrotaline models in which the established PAH can be successfully treated with a variety of agents. Using the SU5416/hypoxia-exposed rat model, the major aims of this study were to (1) examine whether Rho kinase-mediated vasoconstriction contributes to severe end-stage occlusive PAH, (2) address the relationship between occlusive vascular remodeling and vasoconstriction during progression of the disease, and (3) compare the pulmonary vascular responsiveness to different classes of vasodilators.

Materials and Methods

Animals

All experimental procedures were approved by the Animal Care and Use Committee of the University of Colorado at Denver and Health Sciences Center. Adult male Sprague-Dawley rats weighing ~200 g were injected subcutaneously with SU5416 (20 mg/kg; SUGEN Inc), which was suspended in carboxymethylcellulose (0.5% [wt/vol] carboxymethylcellulose sodium, 0.9% [wt/vol] NaCl, 0.4% [vol/vol] polyisorbate, 0.9% [vol/vol] benzyl alcohol in deionized water). The rats were then exposed to chronic hypoxia in a hypobaric chamber (barometric pressure, ~410 mm Hg; inspired O2 tension, ~76 mm Hg) for up to 3 weeks. Some rats were returned to normoxia (altitude of Denver, 1500 m; barometric pressure, ~630 mm Hg; inspired O2 tension, ~120 mm Hg) for an additional 2 weeks. Because it has been shown that the PAH is progressive even after removal of the animals from the hypoxic environment, we studied 2 groups of rats injected with SU5416 and exposed to chronic hypoxia; the first group was studied 2 weeks after SU5416 injection (2-week exposure to hypoxia; early) and the second group was studied 5 weeks after SU5416 injection (3-week exposure to hypoxia and 2 additional weeks at normoxia; late).

Catheterized Rats

Early- and late-stage rats were anesthetized with intramuscular ketamine (100 mg/kg) and xylazine (15 mg/kg) for placement of catheters in the right jugular vein, right ventricle, and right carotid artery. Anesthetized rats were placed in a ventilated plastic box, and right ventricular (RV) systolic pressure (RVSP) and mean systemic arterial pressure (MSAP) were measured with pressure transducers. RVSP instead of pulmonary artery pressure was measured because in the late-stage rats, we could not consistently catheterize the pulmonary artery because of the extremely high pressures. Cardiac output was determined by a standard dye-dilution method, as previously described.

Isolated Perfused Lungs

Lungs were isolated from anesthetized late-stage rats (5 weeks after SU5416 injection) (pentobarbital sodium 30 mg IP) after intracardiac injection of 100 IU of heparin. The techniques of lung isolation, ventilation, and perfusion have been described in detail. Experiments were performed using 20 mL of heparinized blood as the perfusate. This blood was collected by cardiac puncture of adult male normal rats anesthetized with isoflurane (Baxter). The vascular resistance of the blood-perfused late-stage hypertensive lungs was very high, and perfusion rate was arbitrarily set at 0.008 mL/min per g of body weight instead of our usual 0.04 mL/min per gram of body weight for isolated normal and chronic hypoxia–induced hypertensive rat lungs. Isolated lungs were ventilated with 21% O2/5% CO2/74% N2, and blood temperature was kept at 37°C. The drugs described in subsequent experimental protocols were added to the perfusate reservoir to achieve the calculated circulating concentrations.

Pulmonary Artery Count

A quantitative analysis of luminal obstruction was performed by counting at least 200 small pulmonary arteries (OD, ~50 μm) per lung section from each rat in the 2 groups by an investigator who was unaware of the source of the sections. Vessels were assessed for occlusive lesions on hematoxylin/eosin slides and scored as: no evidence of neointimal formation (open); partial (~50%) luminal occlusion; and full-luminal occlusion (closed).

Immunoprecipitation of MYPT1 and Western Blot Analysis

Frozen rat lung tissue was homogenized in a B buffer (20 mmol/L HEPES [pH 7.4], 1 mmol/L dithiothreitol, 10% glycerol, 0.1% Triton X-100). The tissue homogenate was centrifuged at 10 000 rpm for 10 minutes. Protein concentration in the supernatant was determined by Bradford assay using Bradford reagent from Sigma. Whole-lung protein extracts (500 μL; protein concentration, 10 μg/μL) were incubated with 7 μL of anti-MYPT1 antibody (Upstate) for 4 hours at 4°C to allow antibody–antigen complexes to form. Washed and equilibrated EZview Red Protein A Affinity Gel beads (50 μL) (Sigma) were added to antibody–antigen complex and incubated overnight at 4°C with gentle mixing. Beads were pelleted by centrifugation and washed, and antibody–antigen complex was eluted following the protocol of the manufacturer (Sigma). Samples were boiled for 5 minutes and subjected to electrophoresis on 4% to 12% gradient NuPAGE Bis-Tris gels (Invitrogen) and transferred to nitrocellulose membranes. Vessels were visualized using Renaissance Western Blot Chemiluminescence Reagent (NEN Life Science Products) in NuPAGE transfer buffer containing 10% methanol. Prestained molecular mass marker proteins (Bio-Rad) were used as standards for the SDS-PAGE. Western blots were performed for phosphorylated MYPT1 using anti–phospho-MYPT1 (Ser602) (Upstate, New York) and anti–phospho-MYPT1 (Thr696) (New England Biolabs). Western blots were visualized using Renaissance Western Blot Chemiluminescence Reagent (NEN Life Science Products) and estimated by densitometry.

Experimental Protocols
the early and late groups of rats. After baseline hemodynamic measurements, bradykinin (1 and 3 μg per rat) was injected as a bolus into the jugular vein at 10-minute intervals, and the rats were then exposed to 80 parts per million (ppm) NO gas in a ventilated small plastic box for 5 minutes. Fifteen minutes after the end of NO exposure, the rats were given either fasudil (1, 3, and 10 mg/kg, IV bolus) or iloprost (3, 10, 30, and 100 ng/kg per minute, IV infusion at the rate of 0.1 mL/min). In preliminary studies, all rats in the late group (but none in the early group) showed a severe rebound pulmonary hypertension after exposure to NO gas; therefore, we eliminated this step from the protocol for the late group. In addition, 5 of 8 rats in the late group (but again none in the early group) died during the onset of iloprost infusion, presumably because of peripheral systemic vasodilation, illustrating the degree of preload dependency of these hemodynamically compromised animals. At the end of the experiments, rats were euthanized with an overdose of pentobarbital; lungs were collected for histological and protein expression evaluation and hearts for right ventricle/left ventricle + septum (RV/LV+S) weight ratio measurement. In a separate group of late-stage rats, acute effects of the dual endothelin A/B (ET_A/B) receptor antagonist J-104132 were examined. After baseline hemodynamic measurements, J-104132 (1 mg/kg) was injected intravenously. Thirty minutes after J-104132 injection, fasudil (10 mg/kg, IV) was given.

Isolated Blood-Perfused Lungs
In preliminary experiments, we found that with the usual perfusion rate of 0.04 mL/min per gram,20-27 the initial perfusion pressure of isolated blood-perfused lungs from the late-stage rats was extremely high (presumably because of the precapillary occlusive lesions in this model) and that the perfusion pressure continued to rise. (It reached 100 mm Hg within 10 minutes.) We therefore arbitrarily reduced the rate of perfusion to 0.008 mL/min per gram. Even at this low rate, the baseline pressure steadily increased to 50 to 70 mm Hg over 40 minutes. We examined the acute effects of selective ET-1 receptor blockers (dual ET_A/B, J-104132, 1 μmol/L; ET_A, BQ123, 5 μmol/L; Calbiochem) and Y-27632 (10 μmol/L; Calbiochem) on this spontaneous vasoconstriction. Forty minutes after the perfusion was started, J-104132, BQ123, and Y-27632 were added to the perfusate in 15-minute intervals. In lungs from a separate group of late-stage rats, we additionally examined the effects of fasudil (10 μmol/L, added to the perfusate reservoir 40-minute after perfusion was started) on this spontaneous increase in perfusion pressure.

Statistical Analysis
Values are means±SEM. Comparisons between groups were made with Student’s t test or ANOVA with Scheffe’s post hoc test for multiple comparisons. Correlation between occluded vessel density and RVSP or RV/LV+S was assessed by linear regression analysis. Differences were considered significant at P<0.05.

Results
Baseline Hemodynamic Measurements in Catheterized Rats
The early group of rats had high RVSP, with reduced cardiac output and normal MSAP as compared with the historical values of these parameters in control rats in Denver (described previously20 and K.G. Morris, M. Oka, unpublished data, 1993) (Figure 1A through 1C). The late group had an even higher RVSP and lower cardiac output than the early group. These results indicated that the early group had severe PAH, and the late group developed even more severe PAH, comparable to that observed in patients with end-stage PAH.20

RV Hypertrophy
Consistent with the baseline RVSP data, both the early and the late groups of rats developed significant RV hypertrophy, as assessed by the RV/LV+S weight ratio, with a greater severity in the late group (Figure 1D).

Density of Occluded Pulmonary Arteries
As shown in Figure 2A, there were more closed and fewer patent small pulmonary arteries (OD, <50 μm) in the lung sections of the late than of the early group (43±1 versus 28±5% and 44±1 versus 60±3% for closed and open vessel density, respectively; P<0.05). The density of occluded vessels was positively correlated with both RVSP and RV/LV+S weight ratio (Figure 2B and 2C).

Phosphorylation of MYPT1 in Lungs
Figure 3 shows the comparison of MYPT1 phosphorylation levels at the inhibitory site (Thr696) in lung tissue samples. The phosphorylation levels were significantly increased in lungs from both the early- and late-stage rats. Acute fasudil treatment completely reversed the elevated MYPT1 phosphorylation in lungs from the late-stage rats.

Acute Effects of Various Vasodilators on PAH in Catheterized Rats
Bradykinin (3 μg), an endothelium-dependent vasodilator, lowered RVSP significantly in the late (16±2%) but not in the early stage of the disease (4±4%), whereas it elicited similar reductions in MSAP in both stages of PAH (31±4 and 38±4% in the early and late stage, respectively). In the early stage of the disease, inhaled NO (80 ppm) caused an 18±2% reduction (from 51±6 to 41±4 mm Hg) in RVSP but no change in MSAP (105±5 versus 99±4 mm Hg). The very high dose of iloprost (100 ng/kg per minute) elicited a small decrease in both RVSP (from 56±4 to 51±4 mm Hg) and MSAP (from 110±7 to 100±5 mm Hg), which was accompanied by a trend toward an increase in cardiac output (from 0.041±0.006 to 0.058±0.011 L/min; P=0.07). In contrast, fasudil lowered RVSP rapidly (the vasodilator response plateaued within 5 minutes) and markedly in a dose-dependent fashion (from 62±6 to 50±5 and 35±3 mm Hg at 3 and 10 mg/kg, respectively), with no change in cardiac output (Figure 4). Fasudil was not a specific pulmonary vasodilator as it also decreased the MSAP dose-dependently.
(from 92±6 to 70±4 and 46±2 mm Hg at 3 and 10 mg/kg, respectively). Even in the late stage of PAH, with a higher density of occluded pulmonary vessels and more severe pulmonary hypertension, fasudil caused a marked, dose-dependent reduction in RVSP (Figure 4B). Although the percentage of reduction in RVSP by fasudil was comparable in the early and late groups (Figure 4), the absolute post-fasudil (10 mg/kg) RVSP was higher in the late group (58±6 versus 35±3 mm Hg; *P<0.05). This is illustrated in Figure 5, which partitions the hypertension of the 2 groups of rats into fasudil-reversible (vasoconstriction) and fasudil-irreversible (perhaps largely structural remodeling) components. The apparent increase in the remodeling component of the late group coincided with the increased density of occluded small pulmonary arteries in this group (Figure 2). The dual ET₄/ET₅ receptor antagonist J-104132 slightly but significantly reduced the high RVSP in the late group animals (Figure 6A).

**Isolated Blood-Perfused Lungs**

Figure 6B shows a representative perfusion pressure trace of blood-perfused lungs from a late-stage rat. The initial high perfusion pressure spontaneously decreased during the first 10 to 15 minutes of perfusion and then began to rise progressively without any overt signs of lung edema. BQ123 decreased this vigorous spontaneous vasoconstriction gradually, and J-104132 had no further effects. In contrast,
vasodilator testing revealed that this animal model mimics the SU5416/hypoxia-exposed rat model of occlusive pulmonarystriction contributed substantially to the severe PAH of the rats, there was a spontaneous, marked, and progressive increase in the baseline perfusion pressure, which was immediately and dramatically reduced by Y-27632 or fasudil. Inhibition of myosin phosphatase and increased myosin light chain phosphorylation have now been recognized as a major mechanism of smooth muscle contraction via Ca$^{2+}$ sensitization. Rho kinase inhibits myosin phosphatase by phosphorylating its regulatory subunit MYPT1 and thereby induces Ca$^{2+}$ sensitization. Our results showed that phosphorylation of MYPT1 was increased in lungs from both the early- and late-stage rats and that the increased phosphorylation in the late-stage lungs was reversed by fasudil. These observations provide evidence that Rho kinase was in fact activated in this model and that fasudil caused vasodilation by dephosphorylation of MYPT1 via Rho kinase inhibition (although this study does not rule out the possibility that in blood-perfused lungs from the late-stage rats, there was a spontaneous, marked, and progressive increase in the baseline perfusion pressure, which was immediately and dramatically reduced by Y-27632 or fasudil.

Figure 5. RVSP before and after intravenous administration of fasudil (10 mg/kg) in the early-stage (n=7) and the late-stage (n=6) rats. The dashed line indicates the normal values of the difference between before and after fasudil (black area) can be considered as the reversible obstruction (vasoconstriction). The residual hypertension after fasudil treatment (gray area) can be considered as largely attributable to fixed obstruction (structural remodeling). Values are means±SE. *P<0.05 vs respective before value, #P<0.05 vs early after fasudil.

Y-27632 caused an additional rapid and marked reduction of perfusion pressure (Figure 6B and 6C). Fasudil also immediately and markedly reduced the spontaneous vasoconstriction (Figure 6D).

Discussion
This study demonstrated that Rho kinase–mediated vasoconstriction contributed substantially to the severe PAH of the SU5416/hypoxia-exposed rat model of occlusive pulmonary vascular disease. Both hemodynamic evaluation and acute vasodilator testing revealed that this animal model mimics the situation of end-stage, severe angioproliferative PAH in patients, in which a decrease in cardiac output is a predictor of short survival. In addition, it appeared that the conventional vasodilators, such as inhaled NO or intravenous iloprost, agents that are used in acute pulmonary vasodilator testing in patients with PAH, might not be able to unmask the Rho kinase–mediated abnormal vasoconstriction of the severe PAH.

It is generally accepted that over time in PAH, the hypertensive component attributable to acutely reversible vasoconstriction decreases, whereas that attributable to fixed vascular remodeling increases. Indeed, we found in this study that with increasing severity of the hypertension, the density of occlusive pulmonary vascular lesions and the nadir to which the RVSP could be reduced by acute intravenous fasudil increased. However, surprisingly, the absolute contribution of the fasudil-reversible component was not reduced in the late-stage versus early-stage rats (Figure 5). In other words, Rho kinase–mediated sustained vasoconstriction was substantially involved in the elevated RVSP even in the end-stage PAH in this model.

The studies of perfused lungs from the late-stage rats proved that the Rho kinase inhibitors were vigorous pulmonary vasodilators. We found that in blood-perfused lungs from the late-stage rats, there was a spontaneous, marked, and progressive increase in the baseline perfusion pressure, which was immediately and dramatically reduced by Y-27632 or fasudil.

Inhibition of myosin phosphatase and increased myosin light chain phosphorylation have now been recognized as a major mechanism of smooth muscle contraction via Ca$^{2+}$ sensitization. Rho kinase inhibits myosin phosphatase by phosphorylating its regulatory subunit MYPT1 and thereby induces Ca$^{2+}$ sensitization. Our results showed that phosphorylation of MYPT1 was increased in lungs from both the early- and late-stage rats and that the increased phosphorylation in the late-stage lungs was reversed by fasudil. These observations provide evidence that Rho kinase was in fact activated in this model and that fasudil caused vasodilation by dephosphorylation of MYPT1 via Rho kinase inhibition (although this study does not rule out the possibility that
fusudil also inhibited myosin phosphatase via a protein kinase N1 or CPI-17 pathway). Although we believe myosin light chain dephosphorylation and smooth muscle cell relaxation, ie, vasodilation, are the most likely explanations for the fusudil-induced decrease in pulmonary vascular resistance, further studies will be necessary to determine whether inhibition of Rho kinase also decreases vascular resistance by altering smooth muscle cell cytoskeletal organization and/or the patency of the occlusive neointimal lesions.

One important question that should be addressed is what upstream mediators are involved in activating the RhoA/Rho kinase pathway. Because the G protein–coupled receptor agonist ET-1 activates Rhoa/Rho kinase in vascular smooth muscle, and has been implicated in the pathogenesis of several forms of animal model and human PAH, we examined the acute effects of the dual ETα, receptor antagonist J-104132 in anesthetized and catheterized late-stage rats. We found that J-104132 elicited a moderate reduction in RVSP, suggesting that ET-1 contributed to the sustained abnormal pulmonary vasoconstriction. This is consistent with a clinical report that acute administration of bosentan, a dual ETα receptor antagonist, causes significant pulmonary vasodilation in patients with severe PAH. Our in vivo observation was further supported and extended by the findings in isolated blood-perfused lungs from the late-stage rats. We found that the ETα antagonist BQ123 partially reversed the spontaneous abnormal vasoconstriction but that J-104132 had no further effects, indicating that ET-1 acting via ETα receptor is involved in the Rho kinase–mediated vasoconstriction. This agrees with previous studies reporting that the ETα, but not the ETβ receptor is responsible for sustained ET-1–induced Rhoa activation and Rho kinase–mediated MYPT1 phosphorylation. However, it should be emphasized that ET-1 receptor blockade was much less effective than Rho kinase inhibition in reducing the hypertension, and it is likely that additional vasoconstrictor signals converge on the activation of RhoA/Rho kinase.

We tested several different vasodilators in the early group of PAH rats and found that whereas inhaled NO (a cGMP/protein kinase G–dependent vasodilator), intravenous bradykinin (which presumably acts via endothelium-derived NO and/or hyperpolarizing factor), and iloprost (a cAMP-protein kinase A–dependent vasodilator) caused minimal to modest reductions in RVSP, intravenous fusudil reduced it dramatically. Both protein kinase G and protein kinase A can cause Cac2+ desensitization via inhibition of RhoA activation and/or inhibition of MYPT1 phosphorylation. Thus, cyclic nucleotide–dependent vasodilators, such as NO and iloprost, could in theory reverse RhoA/Rho kinase–mediated vasoconstriction. The reason why NO and iloprost were much less effective than fusudil in reducing RVSP in the early group of PAH rats is unclear, but a possible explanation is that phosphodiesterase (such as type 1, 3, and 5) expression/activity could be upregulated in the hypertensive pulmonary circulation and limit the efficacy of cyclic nucleotide–dependent vasodilators. Nevertheless, our results clearly indicate that fusudil could be more effective in reducing high pulmonary blood pressure than the conventional vasodilators in at least some forms of severe PAH.

Accumulating evidence from several laboratories strongly suggests that RhoA/Rho kinase signaling plays a key role in the pathogenesis of various animal models of pulmonary hypertension, including hypoxia-induced, monocrotaline-induced, and shunt-induced pulmonary hypertension and Denver-raised, fawn-hooded rats. Furthermore, a recent preliminary report indicates that there is high RhoA/Rho kinase activity in the small hypertensive pulmonary arteries of transplanted PAH lungs, and we wonder whether Rho kinase inhibitors would more effectively reverse human severe PAH than the conventional vasodilators. Low doses of intravenous fusudil have been administered acutely to 2 small groups of patients with moderate PAH and have been found to cause only slight decreases in pulmonary arterial pressure. Based on our results in rat models of pulmonary hypertension (previous studies and this study), it is likely that higher doses of Rho kinase inhibitor will have to be given to better test the role of Rho kinase–mediated vasoconstriction in human PAH and that they will have to be given via inhalation to avoid systemic vasodilation.

In summary, this study showed that the Rho kinase inhibitor fusudil acutely and effectively reduced the end-stage angioproliferative PAH in rats resembling severe human PAH histologically (presence of occlusion of precapillary vessels by proliferating endothelial cells) and hemodynamically (high RVSP and low cardiac output). It would be informative to test drugs like fusudil (an unconventional potent vasodilator) in patients who lack responsiveness to conventional vasodilators (prostacyclin, NO, or adenosine). The introduction of such drugs into clinical treatment would appear reasonable, because reduction in RV preload would be expected to prolong life in patients with severe, end-stage PAH.

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Disclosures
I.F.M. has served as a consultant for CoTherix Inc.

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