Angiotensin-Converting Enzyme Inhibitor Preserves p21 and Endothelial Nitric Oxide Synthase Expression in Monocrotaline-Induced Pulmonary Arterial Hypertension in Rats

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Background—Pulmonary arterial hypertension (PAH) is associated with structural changes in the pulmonary vasculature characterized by the proliferation of cellular components of the vessels. ACE inhibitor (ACEI) may have beneficial effects in treating PAH, but its precise mechanism of action in the remodeling process is unclear. p21 is a cyclin-dependent kinase inhibitor that may have a protective role in this process by inhibiting cellular proliferation. Endothelial nitric oxide synthase (eNOS) has also been shown to be protective by its vasodilatory effect. Therefore, we investigated whether expression of p21 and eNOS was modulated by ACEI treatment in a rat model.

Methods and Results—Monocrotaline (MCT) was administered to 2 groups of Sprague-Dawley rats fed a high-cholesterol diet, ie, one group received MCT concomitantly with enalapril treatment (MCT\textsuperscript{+/ACEI} rats), and the other group did not receive enalapril (MCT\textsuperscript{+/ACEI} \textsuperscript{-} rats). After 5 weeks, MRI showed right ventricular hypertrophy in MCT\textsuperscript{+/ACEI} \textsuperscript{-} rats. MCT\textsuperscript{+/ACEI} \textsuperscript{+} rats showed a preserved right ventricular morphology. Isolated pulmonary perfusion studies showed that ACEI significantly upregulated NO production, as measured by nitrite levels. Addition of N-methyl-d-glucamine dithiocarbamate–Fe solution, an NO-trapping agent, reversed the basal vasodilatory effect of ACEI in the pulmonary vasculature. Immunoblot analysis showed decreased p21 and eNOS expression in the lung in MCT\textsuperscript{+/ACEI} \textsuperscript{-} rats, whereas their expression was preserved with enalapril treatment.

Conclusions—ACEI suppresses the development of MCT-induced PAH in rats. The mechanism of action might involve the preservation of p21 and eNOS expression. Both p21 and endothelium-derived NO appear to have protective roles in the development of PAH. (Circulation. 2001;104:945-950.)

Key Words: nitric oxide synthase ■ hypertension, pulmonary ■ remodeling ■ angiotensin

Pulmonary arterial hypertension (PAH) is a progressive disease caused by a variety of pulmonary and/or cardiac disorders and may ultimately result in heart failure and death. A diagnostic classification of the various forms of human PAH is proposed,\textsuperscript{1} and according to this, PAH is pathobiologically associated with vascular remodeling of the pulmonary vasculature, including endothelial cell injury, neovascularization of small arteries, smooth muscle cell (SMC) migration and proliferation, and abnormal accumulation of extracellular matrix proteins associated with activation of matrix metalloproteinases. Although heart-lung transplantation remains the only definitive treatment for patients with advanced PAH, an antiremodeling approach to PAH treatment appears to be feasible, for several reasons. First, hypertensive pulmonary artery (PA) from patients with primary pulmonary hypertension (PH) has been demonstrated to be actively remodeled,\textsuperscript{2} and interference with remodeling has been shown to slow or reverse the progress of the disease experimentally.\textsuperscript{3} Second, factors that are likely to promote remodeling, such as ACE, as well as possibly others, are present at the site of active remodeling.\textsuperscript{4} Therefore, antiremodeling treatment may be a potential therapeutic option against various types of PAH.

Endothelial dysfunction caused by endothelial injury is believed to play a key role in the initiation of development of PAH and is reported to be observed in any kind of human PAH.\textsuperscript{1} Among a variety of endothelial functions, we focused our investigation on the production of endothelium-derived nitric oxide (NO) in this study, because NO produced by endothelial NO synthase (eNOS) is a potent vasodilator and is thought to be involved in the regulation of the pulmonary vascular tone. Whether NO contributes to maintaining basal...
pulmonary vascular tone is still controversial; however, NO also suppresses SMC proliferation, and the lack of eNOS has been associated with accelerated vascular remodeling with high flow stress. Impaired availability of NO might also contribute to the pathogenesis of PAH.

p21 is a cyclin-dependent kinase inhibitor that can arrest cell proliferation during the cell cycle by preventing DNA replication in S phase through both p53-dependent and -independent pathways. Recently, Kibbe et al. reported that NO prevents p21 degradation in vascular SMCs, leading to the inhibition of SMC proliferation. Therefore, p21 may have a protective role in the remodeling of the pulmonary vasculature by inhibiting cell proliferation.

We have postulated that reductions in endothelium-derived NO production and decreased p21 expression contribute to pulmonary vascular remodeling. Treatment with ACE inhibitor (ACEI) may inhibit the development of PAH and may also modulate the changes in NO and p21.

Although ACE inhibition has been demonstrated to produce multiple effects within the vascular system, including prevention of angiotensin II formation, potentiation of bradykinin levels, protection of endothelial function, and modulation of NO production, we have focused on a restoration of NO production, and prevention of angiotensin II formation, potentiation of bradykinin levels, protection of endothelial function, and modulation of NO production. We have postulated that reductions in endothelium-derived NO production and decreased p21 expression contribute to pulmonary vascular remodeling. Treatment with ACE inhibitor (ACEI) may inhibit the development of PAH and may also modulate the changes in NO and p21.

**Methods**

**Animal Models**

All protocols and surgical procedures used in this study were approved by the Institutional Animal Care and Use Committee of Carnegie Mellon University. Male Sprague-Dawley (SD) rats, 6 to 7 weeks of age, weighing 150 to 180 g, were purchased from Harlan (Indianapolis, Ind) and fed a high-fat diet containing 1.2% cholesterol, 0.4% cholic acid, and 2.5% olive oil (Dyets).

**MCT Treatment**

MCT (Sigma Chemical Co), dissolved in 1N HCl neutralized with 0.5N NaOH and diluted with PBS, was given as a single subcutaneous injection (40 mg/kg) after 3 weeks of feeding with high-fat food, whereas control rats were injected with the same volume of saline. MCT-injected rats were divided into 2 groups: one group received 4.4 mg · kg⁻¹ · d⁻¹ of an ACEI, enalapril maleate, in their drinking water (MCT/ACEI⁻¹ rats), whereas the other group received drinking water only (MCT/ACEI⁻° rats). This treatment was continued until they were killed at 5 weeks.

**MRI Experiment and Assessment of Right Ventricular Hypertrophy**

For evaluation of right ventricular hypertrophy (RVH) as a result of PAH, multislice spin-echo MRI images were acquired at 8 to 12 time points in a cardiac cycle with respiratory and ECG gating at 2 to 3, 4, and 5 weeks after MCT treatment. After the final MRI studies, the heart and lungs were quickly removed, and some were frozen at −80°C for molecular study or fixed in 3.7% formaldehyde for structural study. Other hearts were excised and weighed. RVH was estimated by measuring the ratios of RV free wall weight to body weight and RV weight to left ventricular (LV) free wall plus interventricular septum weight.

**Isolated Perfused Lung Experiment**

Rats were anesthetized with an injection of 50 mg/kg of sodium pentobarbital IP and were intubated and ventilated with room air with a Harvard volume-controlled ventilator (10 mL/kg, 60 strokes/min). The chest was opened to expose the heart and lungs. Heparin (300 U) was injected into the left ventricle. The animal was exsanguinated by LV puncture. Cannulas were inserted into the left atrium (LA) through the LV and into the main PA through the RV. Perfusion of the lungs was initiated by pumping a steady flow of perfusate into the pulmonary arterial cannula, gradually increasing the rate to 0.06 mL · min⁻¹ · g⁻¹ with a nonrecirculating mode. The heart and lungs remained in situ for the experiment. PA and LA pressures were measured by transducers placed near the cannulas. LA pressure was kept at 0 cm H₂O by adjusting the height of the reservoir. The perfusion apparatus was maintained at 37°C to 38°C. The perfusate, pH 7.4, 37°C, contained (in mmol/L) NaCl 131, KCl 4.7, MgSO₄ 1.2, NaHCO₃ 22.6, KH₂PO₄ 1.2, CaCl₂ 2.5, and glucose 10.0, and 4% BSA (wt/vol), equilibrated with 9% O₂, 5% CO₂. After 20 minutes of stabilization, pressures were recorded for 30 minutes as described previously. Then, the perfusate was changed to one containing an NO-trapping agent, N-methyl-l-glucamine dithiocarbamate (MGD)–Fe solution (10 mmol/L MGD and 2 mmol/L Fe) for 1 minute and switched to the initial perfusate for 20 minutes. MGD was synthesized by the method of Shinobu et al.

Next, the perfusate was changed to one containing an NO donor, S-nitroso-N-acetylpenicillamine (SNAP, 1 mmol/L, Sigma Chemical), for 1 minute and switched again to the initial perfusate for 15 minutes.

**Measurements of Nitrite Levels in Perfusate**

Perfusate was also collected from the LA cannula at the indicated time points during the lung perfusion study. The nitrite levels were measured with a nitrate/nitrite assay kit (Cayman Chemical Co) according to the manufacturer’s protocol.

**Immunoblot Analysis**

Protein extracts were prepared according to the method of Gödecke et al. Protein (60 µg in the in vivo and 100 µg in the in vitro experiments) was separated on SDS-polyacrylamide gels and electroblotted onto a PVDF membrane. p21 and eNOS were detected by monoclonal antibodies (BD). Protein bands were visualized by use of the Supersignal chemiluminescence detection system (Pierce).

**NOS-Inhibitor Experiments**

To determine what effect chronic eNOS inhibition has on the expression of p21 in the lung, some SD rats fed a high-fat diet were treated with a continuous infusion of N-iminoethyl-l-ornithine (L-NIO, 10 mg · kg⁻¹ · d⁻¹; Cayman Chemical Co), a potent NOS inhibitor. This drug was delivered at a rate of 38 µmol · kg⁻¹ · d⁻¹ for 35 days with a miniosmotic pump (Alzet; ALZA Corp) implanted so that the catheter from the pump was positioned in the jugular vein. The pump itself was placed in a subcutaneous pocket on the dorsum of the animal. Control animals received a continuous infusion of saline from the miniosmotic pumps placed in an identical manner. The lungs were excised after 35 days as described above for immunoblot analysis.

**p21 Induction in Cultured Pulmonary Arterial SMCs by NO Donor**

Rat primary cultured pulmonary arterial SMCs (PASMCs) from SD rats fed a high-fat diet (300 to 330 g) were isolated and cultured as previously reported by Platoshyn et al. Isolated PASMCs were divided into 5 groups. Four groups were preincubated for 60 minutes with PD98059 (25 µmol/L), SB203580 (3 and 10 µmol/L), and vehicle, respectively. Then, they were treated with 800 µmol/L of SNAP for 12 hours, followed by immunoblot analysis. The remaining cells served as a control, incubated without any supplement.
Pathological Assays
Samples were embedded in paraffin and processed for 5-μm sections. Hematoxylin-eosin staining and Victoria blue van Gieson staining were performed in the Transplantation Pathology Laboratory of the University of Pittsburgh Medical Center.

Statistical Analysis
The results are presented as mean±SD. The results were analyzed by ANOVA with StatView software (SAS Institute Inc). A value of P<0.05 was considered to be statistically significant.

Results

MCT Treatment
Initially, 2 doses of MCT were tested, 40 and 60 mg/kg. The 60-mg/kg dose, however, resulted in the early death of 6 of 18 animals. Therefore, we used the 40-mg/kg MCT dose in SD rats fed a high-fat diet. This resulted in no mortality, and all treated animals had RVH (Figure 1, B and E), pulmonary vascular neointimal formation, and concentric obliterative lesions (Figure 2) by 5 weeks.

MRI Experiment and Assessment of RVH
Cardiac MRI showed that RVH developed in MCT+/ACEI− rats at 5 weeks of MCT treatment. ACEI showed a suppressive effect against RVH development in MCT+/ACEI− rats (Figure 1, right). RVH was also estimated by calculating the ratio of RV weight against LV+interventricular septum weight and against body weight (Table). By both calculations, ACEI inhibited RVH.

Isolated Perfused Lung Experiment
Isolated pulmonary perfusion studies were carried out to examine the involvement of NO in the regulation of the pulmonary vascular tone in PH. As shown in Figure 3, the NO-trapping agent MGD-Fe increased mean PA pressure (mPAP) in control rats without MCT treatment, whereas administration of the NO donor SNAP decreased mPAP. MCT+/ACEI− rats had a higher baseline mPAP and exhibited no response to the NO-trapping agent. The capacity of these lungs to respond to NO was demonstrated by the decrease in mPAP when SNAP was added to the perfusate. MCT+/ACEI+ rats showed a similar response to both MGD-Fe and SNAP compared with control rats, although mPAP at baseline was higher. These results provide evidence that NO is produced at baseline in the pulmonary vasculature and that basal production is diminished in MCT+/ACEI− rats. This conclusion is also supported by the observation that baseline nitrate/nitrite levels in MCT+/ACEI− rats are significantly lower than the control and correlate with a higher baseline mPAP (Figure 3).

eNOS and p21 Expression in the Lung
Because the initial concentration of nitrate/nitrite in the perfusate during the experiment correlated with the PAP in control rats, we investigated the expression of 2 NOS isoforms, eNOS and inducible NOS (iNOS), in the lung. iNOS expression was not detected in any of the control or experimental groups (results not shown). eNOS expression was observed in the lungs of the control rats and MCT+/ACEI− rats but was not present in the lungs from MCT+/ACEI+ rats (Figure 4, top). MCT+/ACEI− rats showed a clear preserv-
tion of the expression of eNOS after the MCT treatment compared with the expression in the control.

Next, we investigated the expression of cyclin-dependent kinase inhibitor p21. As shown in the lower panel of Figure 4, p21 expression was decreased in the lung of MCT+/ACEI- rats, but this diminished expression was reversed by ACEI. These results suggest that suppressed p21 expression in the lung may be involved in the remodeling of the pulmonary vasculature in developing PAH.

Regulatory Pathway of p21
To determine whether the reduction in eNOS expression might be responsible for the loss of p21 expression, an experiment was carried out with an NOS inhibitor. Rats fed a high-fat diet were randomly assigned to receive either L-NIO, a potent NOS inhibitor, or saline. As shown in Figure 5, top, NOS inhibition markedly suppressed the expression of p21 in the lung compared with the saline group.

NO has been shown to upregulate p21 via the activation of the mitogen-activated protein kinase (MAPK) ERK1/2 in vascular SMCs. To determine whether a similar pathway is involved in our model, cultured PASMCs were exposed to SNAP in the presence or absence of MAPK inhibitors. As shown in Figure 5, bottom, the addition of SNAP to cultured PASMCs induced p21 expression. Whereas the p38 MAPK inhibitor SB203580 showed no inhibitory effect on p21 induction, the ERK1/2 activation inhibitor PD98059 inhibited SNAP-induced p21 expression. This suggests that NO up-regulates p21 expression via the activation of ERK1/2 in our model.

Discussion
This study was undertaken to determine whether reduced NO availability contributes to PH in a rat model of chronic PAH. We have shown that ACEI administration suppresses the development of PAH, in association with preserved expression of eNOS and p21. Both functional and molecular data indicate that NO production is suppressed in this model of PAH and that ACEI, which blocks the development of PAH, preserves eNOS expression. The loss of eNOS expression is associated with a reduction in p21 expression in the lung, and the reduction in p21 expression is mimicked by an NOS inhibitor in animals fed a high-fat diet. Taken together, these observations suggest that a loss of NO and p21 is part of the pathogenesis of the chronic changes seen in PAH induced by MCT and that therapies such as ACEI may function, at least in part, by preserving eNOS expression.

The regulation of basal pulmonary vascular tone by NO has been proposed and investigated; however, conflicting data have been reported. Even in isolated rat perfused lung experiments using NOS inhibitors, there are large discrepancies in the literature. In this study, MGD-Fe was first used in a perfusion lung experiment as an NO trapper. It clearly shows that the basal pulmonary vascular tone in control animals is maintained, at least in part, by NO, but not in PAH lung.

Figure 4. eNOS and p21 expression in lung. Immunoblot shows that eNOS expression is decreased in MCT+/ACEI- lungs, whereas treatment with ACEI recovers expression. p21 expression shows a similar response to treatment. Representative data from 4 separate experiments.

Figure 5. Regulation of p21 expression by NO. Expression of p21 in lung is suppressed by NOS inhibitor L-NIO administration in vivo (top). Expression of p21 in rat pulmonary arterial SMCs is upregulated by NO donor SNAP administration (bottom), p38 MAPK inhibitor SB203580 had no suppressive effect on p21 induction by NO supplement, whereas ERK1/2 activation inhibitor PD98059 suppresses induction of p21. Representative data from 3 separate experiments.
Although the beneficial effect of ACEI against PAH has been investigated experimentally\textsuperscript{22–24} and clinically\textsuperscript{5,25–29} the conclusions are controversial. Explanations for this discrepancy have been ascribed to inadequate dosing, species difference, or differences between inhibiting established lesions in patients and newly developing lesions in animals. In our rat model, we have observed neointimal formation and concentric obliteratorive lesions in the PA, the characteristic pathological findings in human PAH. Previously, rats treated with MCT and pneumonectomy with augmented pulmonary blood flow showed similar pathological findings. As in our model, the investigation shows a similar inhibitory effect of ACEI on pulmonary vascular remodeling.\textsuperscript{24}

Cyclin-dependent kinase inhibitor p21 has been proposed to inhibit vascular SMC proliferation in vascular injury\textsuperscript{9,10} as well as tumor cell proliferation. NO has also been shown to inhibit vascular SMC proliferation in vitro\textsuperscript{30} and in vivo.\textsuperscript{31,32} Now, NO exhibits a similar antiproliferative activity against MCT-induced PAH via p21 preservation. Because iNOS has not been detected in this study, eNOS must be responsible for this NO availability. In this study, NO appears to have not only direct vasodilatory effects in PAH but also inhibitory effects on vascular remodeling via the activation of p21. p21 could be induced by NO via ERK1/2 activation in MCT-induced PAH. Further investigation is necessary, however, to elucidate the mechanisms of how p21 protects against vascular remodeling.

Limitations of This Study
As mentioned previously, this MCT-induced PAH model has no human equivalent. The MCT-induced PAH model, however, is a noninvasive, slowly developing, hemodynamically relevant rat model that mirrors human PAH, resulting in RVH.\textsuperscript{33,34} Although the pathogenesis and pathophysiology of this model cannot be considered identical to all forms of human PAH, the MCT-induced PAH model shows endothelial injury and dysfunction, which is generally seen in human PAH.\textsuperscript{35} We still need to investigate, however, whether our findings in this model can be reproducible in other PAH models.

Because we investigated the involvement of NO and p21 preservation only in the development of PAH in MCT-treated rats, we have no evidence to discuss whether these are involved in a treatment for established PAH by ACEI. Further investigation is necessary to elucidate this.

Conclusions
We propose that the MCT insult against rats fed a high-fat diet, initially causing endothelial cell injury, leading to endothelial cell dysfunction and a decrease in eNOS-derived NO production, might be responsible for the downregulation of the expression of p21, eventually resulting in the structural changes in the pulmonary vessels. The ACEI enalapril has been shown to be able to restore this deleterious pathway by increasing the availability of NO by preserving eNOS expression. Our findings suggest that eNOS and p21 might be potential therapeutic targets for chronic PAH related to cardiopulmonary disorders.

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