Endothelin A Receptor Blockade Improves Nitric Oxide–Mediated Vasodilation in Monocrotaline-Induced Pulmonary Hypertension

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Background—Nitric oxide (NO) and endothelin (ET) have been implicated in the pathogenesis of pulmonary hypertension (PH). Chronic ET A antagonist therapy reduces PH in monocrotaline (MCT)-treated rats. Interactions between the L-arginine–NO pathway and the ET system have been described. We therefore studied the effect of long-term treatment with an oral ET A antagonist (LU 135252) on NO-related vasodilation in isolated lungs from control rats and rats with MCT-induced PH.

Methods and Results—Three weeks after MCT injection, PH was associated with an increase in right ventricular pressure (from 27.4±0.9 to 66.6±4.1 mm Hg) and a decrease in endothelium-independent vasodilation in response to sodium nitroprusside (10−10 to 10−6 mol/L; ΔE max, from 11.1±0.9 to 2.7±0.3 mm Hg). Endothelium-dependent vasodilation in response to acetylcholine (10−9 to 10−4 mol/L) and the calcium ionophore A23187 (10−9 to 10−7 mol/L) remained unaffected. Treatment with LU 135252 did not significantly affect the endothelium-dependent and -independent vasodilations in control rats. However, in MCT-treated rats, LU 135252 therapy significantly reduced right ventricular pressure (39.7±2.1 mm Hg), potentiated acetylcholine-induced vasodilatation (ΔE max, from 1.6±0.2 to 3.7±0.4 mm Hg), and improved the responses to sodium nitroprusside (ΔE max, from 2.7±0.3 to 5.6±0.6 mm Hg). LU 135252 did not significantly alter the non–receptor-mediated endothelium-dependent vasodilation to A23187 or pulmonary constitutive NO synthase activity.

Conclusions—MCT PH is associated with a reduced smooth muscle responsiveness to NO but a maintained endothelium-dependent vasodilatory potency. Long-term ET A antagonist therapy not only restores smooth muscle responsiveness to NO but also increases endothelium-dependent dilation in response to acetylcholine. This mechanism may contribute to the therapeutic benefit of ET A antagonists in PH. (Circulation. 1998;97:2169-2174.)

Key Words: endothelin □ pulmonary heart disease □ endothelium □ drugs

Pulmonary hypertension is associated with vascular endothelial cell metabolic alterations that may contribute to the pathogenic process. Among these, potential modifications in the L-arginine–NO pathway and the ET system have generated much attention not only because of their vascular tone–modulating properties but also because of their potential role in vascular wall remodeling.

The pulmonary circulation is an important site for both clearance and production of the potent vasoconstrictor peptide ET-1.12 ET-1 levels correlate closely with the severity of PH of various etiologies14 with an increase in local ET-1 expression.5 Long-term therapy with specific ET A receptor antagonists reduces the development of PH and right ventricular hypertrophy in both hypoxic and MCT-treated rats5–8 with beneficial effects on pulmonary vascular remodeling.5 In the rat myocardial infarction model, PH caused by congestive heart failure is also ameliorated by long-term application of the specific ET A antagonist BQ-123.9

The mechanism involved in the therapeutic benefits of long-term ET A antagonist therapy in PH has not been studied. The lungs are also rich in ET B receptors, located predominantly on the vascular endothelium10; stimulation of this receptor by ET-1 causes vasodilation through the release of NO and prostacyclin. Inhibition of NOS has been shown to unmask the tonic pressor influence ET-1 in vivo.31 In addition to direct effects through the blockade of the smooth muscle ET A receptor, ET A antagonists theoretically could indirectly modulate endothelial reactivity through unopposed stimulation of the ET B receptor by persistently elevated circulating levels of ET-1. This hypothesis is consistent with the recently described increase in pulmonary pressure by the ET A antagonist RES-701–1 in beagles with dehydromonocrotaline-induced PH (control animals showed no variation).12 Some undefined interactions between the L-arginine–NO pathway and the ET system may be favorably altered by ET A antagonist therapy. Such an interaction has been demonstrated in the
systemic circulation, in which long-term ETα receptor blockade in angiotensin II–induced hypertension was associated with improved endothelium-dependent relaxation to Ach.13

This study was designed to evaluate the effects of long-term therapy with the specific ETα antagonist LU14 on the endothelium-dependent and -independent pulmonary vascular reactivity of rats with MCT-induced PH.

**Methods**

Experiments were carried out on 9-week-old male Sprague-Dawley rats weighing 275 to 375 g (Charles River), which were randomly assigned to receive an intraperitoneal injection of either 0.5 mL of 0.9% saline or 50 mg/kg LU starting 48 hours before the intraperitoneal injection and subsequently for 3 weeks. This dosage regimen of LU has previously been shown effective in the pulmonary vasodilation of rats with MCT-induced PH.

Twenty-four hours after the last gavage, rats were anesthetized with sodium pentobarbital (50 mg/kg IP) followed by 2000 U heparin IP (Sigma Chemical). After stable anesthesia was obtained, the left carotid artery was isolated and incised, and a polyethylene catheter (PE 50; 0.97-mm OD, 0.58-mm ID) was inserted to record arterial pressure. A second catheter (0.97-mm OD, 0.58-mm ID) was inserted into the left atrium through an incision in the left ventricle to collect the effluent in the right ventricle. Another cannula was inserted into the left carotid artery and the pulmonary artery was cannulated through an incision in the right ventricle. The lungs perfusion was initiated by slow infusion of either 1 mL or 0.9% saline or 50 mg/kg LU starting 48 hours before the intraperitoneal injection and subsequently for 3 weeks. This dosage regimen of LU has previously been shown effective in prevention of PH in this model.8 This protocol resulted in the creation of four groups: control + saline (n = 18), control + LU (n = 16), MCT + saline (n = 17), and MCT + LU (n = 15). The MCT was dissolved in 1.0 N HCl, and pH was adjusted to 7.4 with 0.5 N NaOH. The compound LU (Knoll AG) was dissolved in 1.0 N NaOH, and pH was adjusted to 7.4 with 0.5 N HCl.

**Experimental Protocol**

Twenty-four hours after the last gavage, rats were anesthetized with sodium pentobarbital (50 mg/kg IP) followed by 2000 U heparin IP (Sigma Chemical). After stable anesthesia was obtained, the left carotid artery was isolated and incised, and a polyethylene catheter (PE 50; 0.97-mm OD, 0.58-mm ID) was inserted to record arterial pressure. A second catheter (0.97-mm OD, 0.58-mm ID) was advanced into the right ventricle through the right jugular vein for measurement of right ventricular pressure. The position of the catheter was guided by the shape of the pressure tracing displayed on an oscilloscope. The arterial and right ventricular pressures were measured with a polygraph and recorded (model TA4000; Gould). The trachea was cannulated with a tubing connect to a rodent ventilator (Harvard Apparatus) and ventilated with room air with a tidal volume of 1 mL and 2 cm H2O positive end-expiratory pressure. A midline sternotomy was performed to expose the heart and lungs, and the pulmonary artery was cannulated through an incision in the right ventricle. Another cannula was inserted into the left atrium through an incision in the left ventricle to collect the effluent from the lungs. The lungs perfusion was initiated by slow infusion (2.0 mL/min) of Krebs’ solution composed of (in mmol/L): NaCl 120, NaHCO3 25, KCl 4.7, KH2PO4 1.18, MgSO4 1.17, CaCl2 2.5, and glucose 5.5. The Krebs’ solution was bubbled with 95% O2/5% CO2 to maintain pH 7.4. The lungs then were rapidly isolated and suspended in a warm (37°C) water-jacketed chamber to be perfused in a recirculating fashion with Krebs’ solution supplemented with albumin (3%) at 37°C. The pulmonary flow was continuously measured with a Transonic flow probe connected to a Transonic flowmeter (Model 208) and put on the circuit proximal to the pulmonary cannula.

**Evaluation of Pulmonary Vasodilations**

After a 10-minute period of stabilization, a cumulative concentration-response curve was performed with Ach, SNP, or ionophore A23187. In the control + saline, control + LU, and MCT + LU groups, the lungs were perfused at an average constant flow rate of 9.23 ± 0.03 mL/min. In these groups, incremental aliquots of thrombokine analog U46619 were injected into the perfusion circuit to increase the baseline perfusion pressure to 15.2 ± 0.4 mm Hg. In the MCT + saline group, a similar perfusion pressure of 15.0 ± 0.7 mm Hg was reached with a slightly lower constant flow rate of 7.3 ± 0.3 mL/min and without the addition of U46619. The total pulmonary pressure was increased to a similar degree in all groups, but a significantly lower concentration of U46619 was required in lungs from MCT-treated rats that received LU (0.80 ± 0.12 × 10−7 mol/L) than in lungs from both control groups (1.78 ± 0.09 × 10−7 mol/L) (P < 0.01). Once the perfusion pressure had reached a plateau, lungs were vasodilated with Ach, SNP, or ionophore A23187 to obtain a cumulative concentration-response curve. Only one concentration-vasodilation curve was obtained for each lung.

**Evaluation of NO Activity**

Tissue samples were homogenized using a 3:1 vol of homogenizing buffer (10 mmol/L HEPES, 0.32 mmol/L sucrose, 0.1 mmol/L EDTA, 1 mmol/L dithiothreitol, 10 μg/mL leupeptin, 2 μg/mL aprotinin, 1 mg/mL phenylmethylsulfonyl fluoride, final pH 7.2). The homogenized samples were centrifuged at 12,000 rpm at 4°C for 15 minutes, and the supernatants were removed and placed on ice.

Samples were assayed in duplicate to measure the conversion of [14C]-arginine to [14C]-citrulline; 20 μL supernatant was incubated in 100 μL NOS activity buffer (50 mmol/L KH2PO4, 1.2 mmol/L MgCl2, 0.24 mmol/L CaCl2, 50 mmol/L valine, 1 mmol/L l-citrulline, 0.1 mmol/L NADPH, 18 μmol/L L-arginine, 30 μmol/L BH4, 10 58 mol/L FAD, and 2 58 mol/L [U-14C]-l-arginine [no activity]). Samples were incubated for 60 minutes at 37°C. After incubation, the reaction was terminated with the addition of 750 μL cold stop buffer (50 mmol/L HEPES, 5 mmol/L EDTA, pH 5.5). The samples were applied to a 1-mL column of Dowex AG 50W-X8 (Na+ form) and then eluted with 4 mL 9.23% ethanol and stored at −20°C. Ach and SNP (Sigma Chemical) were dissolved in 95% ethanol and stored at −20°C. Ach and SNP (Sigma Chemical) were dissolved in saline and kept in stock solution (−20°C) at a concentration of 10−4 and 10−5 mol/L, respectively.

**Drugs**

U46619 and ionophore A23187 (Sigma Chemical) were dissolved in 95% ethanol and stored at −20°C. Ach and SNP (Sigma Chemical) were dissolved in saline and kept in stock solution (−20°C) at a concentration of 10−4 and 10−5 mol/L, respectively.

**Statistical Analysis**

Differences in concentration-response curves between groups were evaluated with a repeated measures ANOVA followed by a multiple-group comparison. Right ventricular pressures, cNOS activities, Emax values, and −log EC50 values were compared with the use of an ANOVA followed by multiple group comparisons with the use of the Bonferroni correction. EC50 and Emax values were obtained with the use of a five-parameters logistic function with SigmaPlot curve-fitting software. All values were expressed as mean ± SEM unless specified otherwise. Statistical significance was assumed at a level of P < 0.05.

**Results**

**Effect of LU on Right Ventricular and Systemic Pressures**

The effects of the ETα antagonist LU on right ventricular systolic pressure in control and MCT-treated rats are...
presented in Figure 1. Right ventricular pressure in MCT-treated rats (66.6 ± 4.1 mm Hg) was significantly ($P < 0.01$) higher than that in control rats (27.4 ± 0.9 mm Hg). LU therapy did not affect right ventricular pressure in control rats but markedly decreased it after MCT to 39.7 ± 2.1 mm Hg ($P < 0.01$). The mean systemic arterial pressures were not significantly affected in all four groups: control saline (106.9 ± 6.2 mm Hg), control LU (106.7 ± 3.6 mm Hg), MCT saline (100.1 ± 4.0 mm Hg), and MCT LU (105.6 ± 3.8 mm Hg).

**Effect of LU on Pulmonary Endothelium-Independent Vasodilation**

SNP ($10^{-10}$ to $10^{-5}$ mol/L) induced a cumulative concentration-dependent vasodilation in isolated perfused lungs (Figure 2A). The response was markedly lowered with MCT with an $E_{\text{max}}$ value of 2.7 ± 0.3 mm Hg compared with 11.1 ± 0.9 mm Hg in control rats ($P < 0.01$).

LU therapy did not significantly modify the vasodilation curve in response to SNP in control rat lungs ($E_{\text{max}}$, 10.1 ± 1.0 mm Hg) but significantly improved it ($P < 0.05$) after MCT administration, increasing the $E_{\text{max}}$ value to 5.6 ± 0.6 mm Hg.

In both LU-treated groups, the $-\log EC_{50}$ values of SNP that induced pulmonary vasodilation were significantly ($P < 0.01$) increased (control, 8.3 ± 0.1; MCT, 8.6 ± 0.2) compared with groups not treated with LU (control, 7.7 ± 0.1; MCT, 7.7 ± 0.1) (Table).

**Effect of LU on Pulmonary Endothelium-Dependent Vasodilation**

ACh ($10^{-9}$ to $10^{-4}$ mol/L) and calcium ionophore A23187 ($10^{-9}$ to $10^{-7}$ mol/L) induced concentration-dependent vasodilations in isolated rat lungs (Figure 2). ACh-induced vasodilation in MCT rat lungs was not significantly different from that in control rat lungs (Figure 2B). The $E_{\text{max}}$ values for ACh were 1.6 ± 0.2 and 2.1 ± 0.2 mm Hg in MCT-treated and control rat lungs, respectively ($P = 0.25$). LU therapy did not significantly modify the concentration-response curve in control rats, although it tended to improve the response with a greater $E_{\text{max}}$ value (2.6 ± 0.1 mm Hg). Half-maximal responses were not significantly different, with $-\log EC_{50}$ values of 6.7 ± 0.2 and 6.5 ± 0.5 in lungs from control rats not treated and treated with LU, respectively (Table). Lungs from MCT-treated rats that received LU showed a significant ($P < 0.01$) improvement in pulmonary vasodilation in response to ACh with a higher $E_{\text{max}}$ value (3.7 ± 0.4 mm Hg). The $-\log EC_{50}$ values for ACh were 6.8 ± 0.3 and 8.4 ± 0.6 ($P < 0.05$) in lungs from MCT-treated rats and MCT-treated rats that received with LU, respectively (Table).

The responses to A23187 ($10^{-9}$ to $10^{-7}$ mol/L) did not differ significantly among the four groups, although at lower concentrations, higher vasodilations were induced in lungs from MCT-treated rats that received LU (Figure 2C and Table).
Effect of LU on Pulmonary NO Activity
The cNOS activities did not differ significantly between control (NaCl, 0.357 ± 0.081 pmol · mg protein⁻¹ · min⁻¹; LU, 0.428 ± 0.127 pmol · mg of protein⁻¹ · min⁻¹) and MCT-treated rats (NaCl, 0.427 ± 0.144 pmol · mg protein⁻¹ · min⁻¹; LU, 0.356 ± 0.169 pmol · mg of protein⁻¹ · min⁻¹). Similarly, iNOS activity did not vary between groups and was at the limit of detection of the assay with an overall mean of 0.017 ± 0.043 pmol · mg of protein⁻¹ · min⁻¹.

Discussion
The major findings of this study are that (1) although endothelium-independent dilation in response to SNP is reduced in MCT-induced PH, EDNO-dependent dilation is preserved and (2) long-term therapy with the ET₄ antagonist LU improved PH and both endothelium-independent dilation in response to SNP and EDNO-dependent dilation in response to ACh.

Endothelium-Dependent and -Independent Vasodilation in MCT-Induced PH
The results of the present study confirm that MCT-induced hypertensive rats have a marked reduction in SNP-induced pulmonary vasodilation. This suggests a reduced responsiveness of the pulmonary vasculature to NO due to either an increase in basal tone or abnormal vascular remodeling. Although it has been convincingly demonstrated that the response of the hypertensive pulmonary circulation to EDNO donors such as SNP is attenuated, studies of the endogenous endothelial production of NO have yielded conflicting results. Studies in isolated pulmonary arteries from hypoxic and MCT-treated pulmonary hypertensive rats have shown reduced responsiveness to the endothelium-dependent vasodilators, including ACh and A23187, whereas others have suggested an increased basal NO production in the latter model. In intact chronically hypoxic lungs, some have observed a maintained basal and agonist-stimulated endothelial NO activity, whereas others have found that both were increased. Responsiveness to ACh also is maintained in isolated lungs from rats with severe MCT-induced PH. A mild selective increase in the total pulmonary vasodilatory response to the EDNO-dependent agonist arginine vasopressin and unchanged response to the calcium ionophore ionomycin was observed in isolated lungs from MCT-treated rats. We found a mild nonsignificant decrease in the response to ACh and no variation in the response to A23187 in MCT-treated rats. We also measured cNOS activity in whole lung homogenates and found it to be unaffacted by PH. Although this is consistent with our results, it may not be an adequate reflection of local vascular NO activity. Resta et al demonstrated an increase in arterial but not venous cNOS with immunocytochemistry in MCT rats. Our results combined with the above studies consequently confirm that although endothelium-dependent dilation may be attenuated in large isolated pulmonary arteries, it is not significantly affected by MCT-induced PH when intact lungs are evaluated. This is consistent with the previously observed enhancement of vasoconstriction by NOS inhibitors in the hypoxic and MCT-induced hypertensive pulmonary circulation.

Pulmonary vascular rings taken from patients with chronic obstructive pulmonary disease exhibit reduced basal and receptor-mediated endothelium-dependent dilation but preserved responses to the EDNO donor SNP. The magnitude of impairment in endothelium-dependent dilation correlates with the severity of intimal hypertrophy and the degree of hypoxia, suggesting a link between endothelial dysfunction and disease severity. Similarly, pulmonary arterial rings taken from patients with Eisenmenger’s syndrome have a reduced response to ACh, even exhibiting paradoxical vasoconstriction at higher concentrations. Although these observations in large conduit pulmonary arteries are consistent with animal data in similar preparations, there is no general agreement on the pathophysiological role of NO in human PH. Indeed, some have reported a decrease in pulmonary cNOS expression by in situ hybridization, whereas another group reported an increase in cNOS immunoreactivity in the pulmonary vascular endothelium of patients with PH. Our study in isolated rat lungs with MCT-induced PH supports an emerg-
ing pattern in animal models that suggests increases in NOS in the smaller resistance vessels, likely as a mechanism of compensation while endothelium-dependent dilation in larger pulmonary arteries appears to be reduced. The limited data available from human studies are not incompatible with a similar interpretation. More functional human studies with various forms and severities of PH are needed to clarify this issue.

Effect of Chronic ET$_A$ Blockade on Endothelium-Dependent and -Independent Vasodilation

Long-term treatment of MCT-treated rats with an ET$_A$ receptor antagonist reduces the increase in right ventricular pressure and hypertrophy. Similar beneficial effects have been shown in chronically hypoxic rats as well as on the right ventricular function and hemodynamics of the rats after myocardial infarction. Because of the previously shown interactions between the l-arginine/NO pathway and the ET system, we hypothesized that long-term ET$_A$ blockade may improve not only endothelium-independent but also endothelium-dependent dilation in the MCT-induced hypertensive rats.

In the control rats, long-term therapy with the specific ET$_A$ antagonist LU did not modify the vasodilator response to SNP. In MCT-treated rats, however, LU therapy strongly potentiated the response to this agonist. Endothelium-independent dilation in response to SNP was markedly improved by LU therapy with an increase in the E$_{\text{max}}$ value as well as a reduction in the E$_{50}$ value. Long-term therapy with ET$_A$ antagonists can reduce MCT-induced and hypoxic pulmonary artery medial hypertrophy, so part of this improvement could easily be attributed to beneficial remodeling of the pulmonary arteries. However, we have previously shown that LU does not reduce pulmonary medial hypertrophy in arteries of 50- to 150-mm diameter, suggesting other mechanisms may be more important. Consequently, reduced basal vessel tone may also contribute to this improvement because MCT induces early pulmonary hyperresponsiveness to various vasoconstrictors.

The mechanisms by which LU could improve EDNO-dependent dilation are not well established. We measured whole lung cNOS and iNOS activities, which were unaffected by LU therapy. The receptor-independent ionophore A23187 tended to show a lower-threshold concentration response in MCT-treated animals but no differences in the E$_{50}$ and E$_{\text{max}}$ values. Together, these results suggest LU therapy did not modify the maximal capacity of the pulmonary vasculature to generate NO from l-arginine. It, however, greatly increased receptor-dependent NO release by ACh in MCT-treated animals with a net tendency for an increase even in the control animals. These suggests an upregulation or increased sensitivity of the endothelial ACh receptor in the pulmonary circulation. Our findings are consistent with those of d’Uscio et al showing that long-term ET$_A$ antagonist therapy improved ACh-dependent dilation in the aortas of rats with angiotensin II–induced systemic hypertension.

The present study, in isolated lungs, has the advantage of being an evaluation of the functional behavior of the whole pulmonary circulation (including large and small vessels) with good control on concentrations of the pharmacological agents used and without effects of variables such as heart rate and systemic pressure. The lungs were preconstricted as needed with U46619 to obtain similarly elevated perfusion pressures of ~15 mm Hg. U46619 was not needed for the MCT+saline group, whereas an intermediate dose was needed for the MCT+LU group. This methodology has the advantage of comparing all groups with a similar baseline vascular tone, but we cannot exclude that the various doses of U46619 may have altered the reactivity to the various agonists that were used. Indeed, a greater dose of U46619 would favor a greater active and reversible component of vascular tone. Preconstriction was not used in the MCT+saline group because hydrostatic pulmonary edema would invariably develop after infusion of this constrictor. However, the observation that despite greater active pharmacological preconstriction the response to the endothelium-dependent dilator calcium ionophore was similar and the response to ACh was less in the control+saline group than in the MCT+LU groups strengthens our conclusion.

In conclusion, we demonstrated that EDNO-dependent dilation is maintained in MCT-induced PH and SNP dilator potency is greatly reduced. Long-term ET$_A$ antagonist therapy improves PH in this model and improves pulmonary vascular reactivity by increasing both endothelium-independent dilation in response to SNP and EDNO-dependent dilation in response to ACh. These findings demonstrate that the ET$_A$ receptor contributes to the development of PH and to alterations of pulmonary vascular reactivity during sustained elevation of the pulmonary pressure and support the development of ET$_A$ antagonists for the therapy of PH.

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References

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