Contribution of Endogenous Endothelin-1 to the Progression of Cardiopulmonary Alterations in Rats With Monocrotaline-Induced Pulmonary Hypertension

Takashi Miyauchi, Ryosuke Yorikane, Satoshi Sakai, Takeshi Sakurai, Megumu Okada, Masaru Nishikibe, Mitsuo Yano, Iwao Yamaguchi, Yasuro Sugishita, Katsutoshi Goto

Endothelin-1 (ET-1) is known to have potent contractile and proliferative effects on vascular smooth muscle cells and is known to induce myocardial cell hypertrophy. We studied the pathophysiological role of endogenous ET-1 in rats with monocrotaline-induced pulmonary hypertension. Four-week-old rats were given a single subcutaneous injection of 60 mg/kg monocrotaline (MCT rats) or saline (control rats) and were killed after 6, 10, 14, 18, and 25 days. In the MCT rats, right ventricular systolic pressure progressively increased and right ventricular hypertrophy developed in a parallel fashion. The venous plasma ET-1 concentration also progressively increased, and this increase preceded the development of pulmonary hypertension. The isolated pulmonary artery exhibited a significantly weaker response to ET-1 in the MCT rats on day 25 but not on days 6 and 14. In the MCT rats, the expression of prepro ET-1 mRNA as measured by Northern blot analysis significantly increased in the heart on days 18 and 25, whereas it gradually decreased in the lungs. The peptide level of ET-1 in the lungs also significantly decreased in the pulmonary hypertensive stage. The expression of prepro ET-1 mRNA had increased by day 6 only in the kidneys. Continuous infusion of BQ-123, a selective ET₄ receptor antagonist, by an osmotic minipump (14.3 mg per day per rat for 18 days) significantly inhibited the progression of both pulmonary hypertension (right ventricular systolic pressure, 77.8±4.2 [mean±SEM] mm Hg [n=10] versus 52.3±2.4 mm Hg [n=7]; P<.01) and right ventricular hypertrophy (right ventricle/left ventricle/septum), 0.56±0.03 [n=10] versus 0.41±0.02 [n=7]; P<.01). Histological examination revealedler that BQ-123 also effectively prevented pulmonary arterial medial thickening. The inhibition of right ventricular hypertrophy by BQ-123 may be partly ascribed to the blockade of excessive stimulation of the heart by ET-1, in addition to the prevention of pulmonary hypertension. The present findings suggest that endogenous ET-1 contributes to the progression of cardiopulmonary alterations in rats with MCT-induced pulmonary hypertension. (Circ Res. 1993;73:887-897.)

KEY WORDS • endothelin-1 • pulmonary hypertensive rats • gene expression • ET₄ receptor antagonist • plasma concentration

Various cardiac diseases (eg, congenital heart disease, valvular heart disease, and left ventricular failure) and pulmonary diseases (eg, emphysema, lung fibrosis, and obstructive airway disease) are often accompanied by pulmonary hypertension, and the severity of pulmonary hypertension is one determinant of the prognosis of such patients. Although some researchers believe that pulmonary vasoconstriction plays a role in the pathogenesis of pulmonary hypertension, the mechanism for the progression of pulmonary hypertension is still poorly understood.

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Endothelin-1 (ET-1), a potent endothelium-derived vasoconstrictor peptide, was recently identified. This peptide induces proliferation of vascular smooth muscle cells. ET-1 has several properties suggestive of a potential pathophysiological role in pulmonary hypertension. First, ET-1 contracts isolated pulmonary vessels and increases pulmonary vascular resistance. Second, ET-1 has a mitogenic effect on vascular smooth muscle cells and fibroblasts consistent with a role in vascular remodeling, a prominent finding in pulmonary hypertensive stages. ET-1 has also been reported to be produced by nonvascular tissues such as the heart, kidneys, and central nervous system. It has been demonstrated that prepro ET-1 mRNA is expressed in cultured rat cardiomyocytes and that ET-1-like immunoreactivity exists in the renal cortex and medulla of rats. In the heart, ET-1 induces myocardial cell hypertrophy and has positive inotropic and chronotropic effects.

A single subcutaneous injection of monocrotaline (MCT), a pyrrolizidine alkaloid, causes pulmonary hy-
pertension in rats.\textsuperscript{19–21} Pathological changes that occur in the lungs after MCT administration consist of vascular endothelial cell damage,\textsuperscript{22} medial thickening of the muscular pulmonary arteries, and neomuscularization of nonmuscular distal arteries.\textsuperscript{20,23} A progressive rise in pulmonary arterial pressure and the development of right ventricular (RV) hypertrophy appear to correlate with these pulmonary vascular structural changes.\textsuperscript{20,23} Although various studies concerning this animal model of pulmonary hypertension have been performed, its etiology is still unknown.

In the present study, the pathophysiological role of endogenous ET-1 was studied from the viewpoint of its contribution to the development and/or progression of cardiopulmonary changes in rats with MCT-induced pulmonary hypertension (MCT rats). Venous plasma and lung ET-1 levels, the expression of prepro ET-1 mRNA in various organs, and pulmonary vascular responses to ET-1 were studied in MCT rats at various disease stages. Furthermore, we studied the effects of continuous infusion of BQ-123, a novel selective ET\textsubscript{A} receptor antagonist,\textsuperscript{24} on the extent of cardiopulmonary changes in MCT rats.

Materials and Methods

Animals

Four-week-old male Wistar rats were used. Treatment with MCT (Wako Pure Chemical, Osaka, Japan) was performed as previously described\textsuperscript{23}; rats were given a single subcutaneous injection of 60 mg/kg MCT (MCT rats) or saline (control rats) and were killed after 6, 10, 14, 18, or 25 days.

On the day of the experiment, each rat was artificially ventilated under anesthesia with sodium pentobarbital (50 mg/kg IP). The thorax was opened, and a heparin-filled needle, connected to a pressure transducer (model SCK-590, Gould, Cleveland, Ohio), was inserted into the RV. RV pressure was recorded by means of a polygraph system (AP-601G amplifier and WT-687G thermal pen recorder, Nihon Kohden, Tokyo, Japan). A blood sample was collected from the RV for determining the plasma ET-1 concentration.

The main pulmonary artery, the aorta, the heart, the lungs, and the left kidney were removed. The heart was divided into the RV, left ventricle (LV), septum (SEP), right atrium (RA), and left atrium (LA), and each portion was separately weighed and rapidly frozen in liquid nitrogen. The lungs and the left kidney were weighed and were also rapidly frozen. The main pulmonary artery and the aorta were immediately placed in Krebs-Ringer solution.

Sandwich Enzyme Immunoassay to Determine Plasma and Lung ET-1 Levels

Each blood sample was placed in chilled tubes containing aprotinin (300 kallikrein inhibiting units/mL) and EDTA (2 mg/mL) and was centrifuged at 2000 g for 15 minutes at 4°C. The plasma was stored at −30°C until used. Plasma (1 mL) was acidified with 3 mL of 4% acetic acid, and immunoreactive ET-1 was extracted with a Sep-pak C-18 cartridge (Waters Associates, Milford, Mass) as previously described.\textsuperscript{25} The eluates were reconstituted with 0.25 mL of assay buffer and subjected to sandwich enzyme immunoassay (EIA) for ET-1.

Sandwich EIA for ET-1 was carried out as previously described using immobilized mouse monoclonal antibody AwETN40, which recognizes the N-terminal portion of ET-1, and peroxidase-labeled rabbit anti-ET-1 C-terminal peptide (15–21)Fab\textsuperscript{25,26} The assay for ET-1 did not cross-react with ET-3 or big ET-1 (cross-reactivity, <0.1%). The detection limit of this EIA was 0.4 pg/mL.\textsuperscript{25,26}

The lung ET-1 level was determined as previously described.\textsuperscript{27} Briefly, the lung, which was frozen in liquid nitrogen and stored at −80°C, was homogenized with a Polytron homogenizer for 60 seconds in 10 vols of 1 mol/L acetic acid containing 10 μg/mL peptatin (Peptide Institute, Osaka, Japan) and immediately boiled for 10 minutes. The homogenate was centrifuged at 20 000 g for 30 minutes at 4°C, and the supernatant was stored at −30°C until used. The supernatant was subjected to sandwich EIA for ET-1.

Measurement of the Vascular Response to ET-1

The main pulmonary artery and the aorta from the rats of both groups were immediately excised and placed in Krebs-Ringer solution of the following composition (mmol/L): NaCl, 113; KCl, 4.8; MgSO\textsubscript{4}, 1.2; CaCl\textsubscript{2}, 2.2; KH\textsubscript{2}PO\textsubscript{4}, 1.2; NaHCO\textsubscript{3}, 25; and glucose, 5.5. After the vessel was freed from the surrounding connective tissues, a 4-mm-long vessel ring (ie, pulmonary artery or aorta) was mounted in a silicon-coated 30-mL organ bath by means of two metal holders, one being anchored and the other being connected to a force displacement transducer (model TB-612T, Nihon Kohden) for the measurement of isometric contractions. The solution was constantly bubbled with a mixture of 95% O\textsubscript{2}–5% CO\textsubscript{2} and kept at 37°C. The resting tension was adjusted to 1.0 g. After equilibration for 2 hours, the response to K\textsuperscript{+} (50 mmol/L) was repeatedly measured at intervals of 30 minutes until a steady response was obtained (three or four times). The dose-response relation for ET-1 (Peptide Institute) was determined by means of cumulative application. The arterial segments were gently treated so as not to injure the intimal surface, and the presence of endothelium was confirmed by the dilator response to acetylcholine (10\textsuperscript{–6} mol/L). The cross-sectional area of each pulmonary artery and aorta was determined from its tissue wet weight and diameter.\textsuperscript{28} The contractile tension was divided by the cross-sectional area for the normalization of the data.

Northern Blot Analysis for Prepro ET-1 mRNA

Total RNA from rat tissues (RV, LV, SEP, LA, RA, lung, kidney) was prepared by selective precipitation in 3 mol/L LiCl and 6 mol/L urea.\textsuperscript{13} Five or 10 μg per lane of total RNA prepared from these tissues was separated by formaldehyde/1.1% agarose gel electrophoresis and transferred onto nylon membranes (Hybond N, Amersham). The membranes were prehybridized at 42°C for 3 hours in a solution containing 1 mol/L NaCl, 50% formamide, 1% sodium dodecyl sulfate, and 150 μg/mL fragmented salmon sperm DNA and were then hybridized with 32P-labeled cDNA probes in the same solution at 42°C for 16 hours. The filters were finally washed in 0.1 x standard saline citrate/0.1% sodium dodecyl sulfate at 50°C and subjected to autoradiography. The prepro ET-1 cDNA used as a probe in the present study was a previously described full-length insert of ArET1-213 that
was labeled by random priming with \([\alpha^{32}\text{P}]{\text{dCTP}}\) (3000 Ci/mmol, Amersham). To normalize the prepro ET-1 signals for the loaded amounts and transfer efficiencies, the same membranes were rehybridized with a \(\beta\)-actin cDNA probe as the internal control.

**Effects of Continuous BQ-123 Infusion on Pulmonary Hypertension and RV Hypertrophy and on the Alteration of Lung Vascular Morphology in MCT Rats**

To investigate the effects of continuous BQ-123 infusion on the MCT rats, osmotic minipumps (model 1701, Alza Corp, Palo Alto, Calif) were subcutaneously implanted in 4-week-old male Wistar rats on the day of injection of 60 mg/kg MCT or saline. BQ-123 was dissolved in saline. The content of the osmotic minipumps was saline alone or saline containing BQ-123. The rats were divided into the following four groups: group I, MCT rats receiving continuous BQ-123 infusion (7.1 mg per day per rat) for 18 days; group II, MCT rats receiving continuous BQ-123 infusion (14.3 mg per day per rat) for 18 days; group III, MCT rats receiving continuous saline infusion for 18 days; and group IV, control rats receiving continuous saline infusion for 18 days.

Eighteen days after the injection of MCT or saline, the rats were killed under pentobarbital anesthesia (50 mg/kg). The hemodynamic parameters were measured, and the wet weight of each heart was measured.

To histologically investigate the lung, the lungs were excised and immersed in formalin. Paraffin sections of 4-\(\mu\)m thickness from the middle region of the left and right lung were stained with hematoxylin and eosin and were examined under light microscopy.

Statistical analysis of the medial wall thickness was performed in accordance with the method described by Ono and Voelkel.\(^{29}\) In brief, measurements of the external diameter and medial wall thickness were made on 30 muscular arteries (in the size ranges of 25 to 50, 51 to 100, and 101 to 200 \(\mu\)m in external diameter) per one lung section. For each artery, the medial wall thickness was expressed as follows: \(\%\) wall thickness=\([(\text{medial thickness}\times 2)/\text{external diameter}]\times 100.\) One lung section was obtained from one rat. For this analysis, seven sections were obtained from each group.

**Statistics**

Data were expressed as mean±SEM. Group means were compared using an unpaired Student’s \(t\) test or one-way analysis of variance with multiple comparisons where appropriate. A value of \(P<.05\) was accepted as statistically significant.

**Results**

**Development of Pulmonary Hypertension and RV Hypertrophy in MCT Rats**

A single injection of MCT caused a progressive increase in RV systolic pressure that was significantly higher than the control value by day 14, after which it rose quite rapidly (Fig 1A). MCT also caused a progressive increase in the ratio of RV to LV+SEP from days 14 to 25 that was significantly greater than the control value, indicating the development of RV hypertrophy in the MCT rats (Fig 1B). Mean central venous pressure was significantly elevated on days 18 and 25 in the MCT rats (Table 1). Mean blood pressure was significantly lower in the MCT than in the control rats only on day 25.

**Table 1. Hemodynamic Measurements**

<table>
<thead>
<tr>
<th></th>
<th>Mean BP, mm Hg</th>
<th>Mean CVP, mm Hg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C (n=16)</td>
<td>101.3±2.3</td>
<td>2.0±0.2</td>
</tr>
<tr>
<td>MCT (n=13)</td>
<td>111.0±7.5</td>
<td>2.4±0.3</td>
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<tr>
<td>Day 10</td>
<td></td>
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<tr>
<td>C (n=9)</td>
<td>111.4±2.8</td>
<td>...</td>
</tr>
<tr>
<td>MCT (n=9)</td>
<td>101.7±5.4</td>
<td>...</td>
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<tr>
<td>Day 14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C (n=20)</td>
<td>110.8±3.3</td>
<td>1.7±0.3</td>
</tr>
<tr>
<td>MCT (n=18)</td>
<td>105.9±4.0</td>
<td>1.7±0.2</td>
</tr>
<tr>
<td>Day 18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C (n=7)</td>
<td>119.1±6.6</td>
<td>2.1±0.4</td>
</tr>
<tr>
<td>MCT (n=8)</td>
<td>104.4±5.7</td>
<td>5.1±0.8*</td>
</tr>
<tr>
<td>Day 25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C (n=21)</td>
<td>118.0±4.0</td>
<td>2.1±0.2</td>
</tr>
<tr>
<td>MCT (n=16)</td>
<td>93.3±7.7*</td>
<td>7.0±0.7*</td>
</tr>
</tbody>
</table>

BP indicates blood pressure; CVP, central venous pressure; C, control rats; and MCT, monocrotaline-injected rats. Values are mean±SEM.

\(*P<.01\) vs the corresponding value in C rats.
TABLE 2. Wet Weight of Hearts and Lungs of Control Rats and Monocrotaline-Injected Rats

<table>
<thead>
<tr>
<th>Day</th>
<th>BW, g</th>
<th>LV+SEP, mg</th>
<th>RV, mg</th>
<th>(LV+SEP)/BW, mg/g</th>
<th>RV/BW, mg/g</th>
<th>Lungs, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C (n=16)</td>
<td>145.0±4.8</td>
<td>356.3±17.0</td>
<td>101.5±14.7</td>
<td>2.69±0.09</td>
<td>0.87±0.03</td>
<td>1.11±0.14</td>
</tr>
<tr>
<td>MCT (n=13)</td>
<td>134.4±4.9</td>
<td>332.9±25.7</td>
<td>105.7±4.8</td>
<td>2.63±0.07</td>
<td>0.85±0.04</td>
<td>1.03±0.09</td>
</tr>
<tr>
<td>Day 10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C (n=9)</td>
<td>157.0±5.4</td>
<td>411.1±13.4</td>
<td>128.9±4.2</td>
<td>2.63±0.09</td>
<td>0.83±0.03</td>
<td>1.33±0.08</td>
</tr>
<tr>
<td>MCT (n=9)</td>
<td>129.8±4.2</td>
<td>347.8±20.5†</td>
<td>116.7±5.8</td>
<td>2.68±0.15</td>
<td>0.90±0.05</td>
<td>1.76±0.16†</td>
</tr>
<tr>
<td>Day 14</td>
<td></td>
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<td></td>
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<tr>
<td>C (n=20)</td>
<td>208.1±6.7</td>
<td>492.3±14.4</td>
<td>166.2±4.6</td>
<td>2.55±0.08</td>
<td>0.86±0.04</td>
<td>1.44±0.11</td>
</tr>
<tr>
<td>MCT (n=18)</td>
<td>178.3±6.7</td>
<td>420.0±11.7*</td>
<td>183.1±11.5</td>
<td>2.59±0.08</td>
<td>1.12±0.05*</td>
<td>2.28±0.22*</td>
</tr>
<tr>
<td>Day 18</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>C (n=7)</td>
<td>243.3±4.2</td>
<td>561.4±16.1</td>
<td>175.7±5.7</td>
<td>2.31±0.05</td>
<td>0.72±0.03</td>
<td>1.17±0.05</td>
</tr>
<tr>
<td>MCT (n=8)</td>
<td>185.6±4.5*</td>
<td>486.3±10.2†</td>
<td>325.0±15.5*</td>
<td>2.63±0.05*</td>
<td>1.75±0.06*</td>
<td>1.99±0.11*</td>
</tr>
<tr>
<td>Day 25</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C (n=21)</td>
<td>291.7±5.3</td>
<td>612.7±13.6</td>
<td>179.2±5.5</td>
<td>2.09±0.04</td>
<td>0.61±0.02</td>
<td>1.37±0.06</td>
</tr>
<tr>
<td>MCT (n=16)</td>
<td>218.8±7.5*</td>
<td>515.0±12.1*</td>
<td>363.9±15.9*</td>
<td>2.32±0.05*</td>
<td>1.63±0.06*</td>
<td>2.15±0.09*</td>
</tr>
</tbody>
</table>

BW indicates body weight; LV, left ventricle; SEP, septum; RV, right ventricle; C, control rats; and MCT, monocrotaline-injected rats. Values are mean±SEM.

*P<.01 and †P<.05 vs the corresponding value in C rats.

(Table 1). The body weight of the MCT rats was significantly lower than that of the control rats on days 10, 14, 18, and 25 (Table 2). However, the lung wet weight of the MCT rats was significantly higher than that of the control rats on days 10, 14, 18, and 25 (Table 2), suggesting the development of inflammatory injury in the lungs of the MCT rats.

**Plasma ET-1 Concentrations**

The plasma ET-1 concentration of the MCT rats progressively increased and was significantly higher than that of the control rats from day 10 (Fig 2). Therefore, the increase in plasma ET-1 concentration preceded the development of pulmonary hypertension. On days 18 and 25, the plasma ET-1 concentration of the MCT rats was markedly elevated (Fig 2).

**Vascular Response to ET-1**

In the ring preparation of the pulmonary artery, ET-1 produced dose-dependent vasoconstriction in both the control and MCT rats. In the MCT rats on day 14, ET-1 at low doses (approximately 10^-10 to 10^-9 mol/L) produced an oscillation-like response (Fig 3B) that was observed in seven of eight rats. However, this oscillation-like response was not observed in the MCT rats on either day 6 or day 25 (data not shown). An oscillation-like response was not observed in the control rats at any stage (ie, on days 6, 14, and 25) (Fig 3A). The doseresponse curves for the vasoconstrictive response to ET-1 did not differ significantly between the MCT and control rats on either day 6 or day 14 (Fig 4, Table 3). On day 25, both the maximal response and the PD2 value (negative logarithm of concentration producing
50% of maximal contraction) were significantly smaller in the MCT than in the control rats (Fig 4 and Table 3), indicating that the pulmonary vascular response to ET-1 was significantly reduced in the MCT rats.

In the ring preparation of the aorta, the dose-response curves for the vasocontractile response to ET-1 did not differ significantly between the MCT and control rats on day 25 (Fig 4 and Table 3).

Expression of Prepro ET-1 mRNA in Various Tissues

The expression of prepro ET-1 mRNA was altered in the various tissues of the MCT rats. Typical examples on days 14 and 25 are shown in Fig 5. Fig 6 depicts densitometric analysis of this blot corrected for β-actin mRNA, which was used as normalization for a constitutively expressed message.

In the MCT rats, prepro ET-1 mRNA expression in the lungs was significantly reduced on days 14 and 25 (Figs 5 and 6), whereas that in the kidneys was significantly increased on days 6, 14, and 25 (Fig 6). Prepro ET-1 mRNA expression in both the RV and LV was significantly increased on days 18 (Fig 6) and 25 (Figs 5 and 6) in the MCT rats. However, prepro ET-1 mRNA expression in either RV or LV did not differ significantly between the MCT and the control rats on day 14 (Figs 5 and 6). On day 25, the prepro ET-1 mRNA expression in SEP, RA, and LA in the MCT rats was also markedly increased (Fig 5).

Lung ET-1 Level

On day 14, the lung ET-1 level of the MCT rats (3.75±0.74 ng/g lung) was significantly lower than that of the control rats (6.47±0.51 ng/g lung, P<.05). On day 25, the lung ET-1 level of the MCT rats (2.69±0.78 ng/g lung) was also significantly lower than that of the control rats (5.74±1.00 ng/g lung, P<.05).

Effects of Continuous BQ-123 Infusion on Pulmonary Hypertension, RV Hypertrophy, and the Alteration of Lung Vascular Morphology in MCT Rats

Continuous infusion of BQ-123 (7.1 mg per day per rat) for 18 days significantly reduced RV systolic pressure (Fig 7) and significantly ameliorated RV hypertrophy (Fig 7 and Table 4). Continuous infusion of BQ-123 at a higher dose (14.3 mg per day per rat) for 18 days was more effective at ameliorating both pulmonary hypertension and RV hypertrophy (Fig 7 and Table 4).

Histology of the lungs of the MCT rats revealed an increase in pulmonary arterial medial thickness (Figs 8B and 9). Continuous infusion of BQ-123 (14.3 mg per day per rat) significantly inhibited the observed increase in the medial thickness of the pulmonary arteries of the MCT rats (Figs 8C and 9).

Discussion

The present study showed that, after treatment with MCT, pulmonary hypertension in rats developed significantly by day 14 and markedly after day 18. An important finding was that the plasma ET-1 level also progressively increased. This increase preceded the development of pulmonary hypertension, suggesting
that ET-1 may be involved in the pathogenesis of pulmonary hypertension in the MCT rats. The elevated plasma ET-1 level did not originate in the lungs, however, since both the peptide level of ET-1 and the expression of prepro ET-1 mRNA in the lungs were significantly lower in the MCT than in the control rats at all stages of pulmonary hypertension. The expression of prepro ET-1 mRNA was already significantly elevated on day 6, i.e., in the prehypertensive stage, only in the kidneys. Therefore, the increased plasma ET-1 level seen at the prehypertensive stage in the MCT rats may be partly attributed to increased ET-1 production in the

TABLE 3. PD₂ Value and Maximum Response to Endothelin-1 in Pulmonary Artery and Aorta

<table>
<thead>
<tr>
<th>Pulmonary artery</th>
<th>Control</th>
<th>MCT</th>
<th>P</th>
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<tbody>
<tr>
<td>Day 6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max response, g/mm²</td>
<td>0.84±0.09</td>
<td>0.86±0.04</td>
<td>NS</td>
</tr>
<tr>
<td>PD₂, -log ED₅₀</td>
<td>8.88±0.08</td>
<td>8.69±0.07</td>
<td>NS</td>
</tr>
<tr>
<td>Day 14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max response, g/mm²</td>
<td>0.91±0.05</td>
<td>0.79±0.09</td>
<td>NS</td>
</tr>
<tr>
<td>PD₂, -log ED₅₀</td>
<td>8.98±0.03</td>
<td>9.04±0.07</td>
<td>NS</td>
</tr>
<tr>
<td>Day 25</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max response, g/mm²</td>
<td>0.65±0.03</td>
<td>0.27±0.02</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>PD₂, -log ED₅₀</td>
<td>8.71±0.07</td>
<td>8.37±0.06</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Aorta</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 25</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max response, g/mm²</td>
<td>0.50±0.01</td>
<td>0.45±0.02</td>
<td>NS</td>
</tr>
<tr>
<td>PD₂, -log ED₅₀</td>
<td>8.76±0.05</td>
<td>8.69±0.09</td>
<td>NS</td>
</tr>
</tbody>
</table>

MCT indicates monocrotaline-injected rats; PD₂, negative logarithm of concentration producing 50% of maximal contraction; and NS, not significant. Values are mean±SEM.

![Image of Northern blot analysis](http://circres.ahajournals.org/)

**Fig 5.** Typical examples of Northern blot analysis are shown for the levels of prepro endothelin-1 (ET-1) mRNA in the heart and the lungs of control (C) rats and monocrotaline (MCT)-injected rats on day 14 (top) and day 25 (bottom). To normalize the prepro ET-1 signals for the loaded amounts and transfer efficiencies, β-actin mRNA levels were compared as the internal control. The prepro ET-1 mRNA (2.3 kb) and β-actin mRNA (2.0 kb) are indicated by arrows. LV indicates left ventricle; RV, right ventricle; SEP, septum; LA, left atria; and RA, right atria. Ten micrograms per lane of total RNA prepared from these tissues, except for the LA and RA of rats on day 14, was loaded. Five micrograms per lane of total RNA from the LA and RA of rats on day 14 was loaded. On day 14, the expression of prepro ET-1 mRNA in the LV, RV, SEP, LA, and RA of the MCT rats was not different from that of the C rats, whereas the expression of prepro ET-1 mRNA in the lungs of the MCT rats was decreased. On day 25, the expression of prepro ET-1 mRNA in the LV, RV, SEP, LA, and RA of the MCT rats was markedly increased, whereas that in the lungs was decreased.
Kidneys. Since MCT causes nephrotoxicity, the increase in prepro ET-1 mRNA expression in the kidneys might be due to a direct action of MCT on the kidneys. The expression of prepro ET-1 mRNA in both the RV and LV in the MCT rats significantly increased at the severe pulmonary hypertensive stage, i.e., on days 18 and 25, suggesting that the marked increase in plasma ET-1 observed at this stage may be partly ascribed to the increase in ET-1 production in the heart as well as in the kidneys. Since circulating ET-1 is mainly eliminated by uptake into the lungs and the kidneys, a decrease in ET-1 elimination by the lungs due to inflammatory injury of the lung may also contribute to the marked increase in plasma ET-1.

Although the dose-response curves for ET-1 in the pulmonary artery did not differ between the control and

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**Fig. 6.** Bar graphs show the level of expression of prepro endothelin-1 (ET-1) mRNA in various tissues from control (C) rats and monocrotaline (MCT)-injected rats. The autoradiogram from Northern blot analysis was scanned by a densitometer, and the ratio between the levels of prepro ET-1 mRNA and β-actin mRNA was calculated. The data for lung, kidney, right ventricle (RV), and left ventricle (LV) of the C rats and MCT rats at various stages are shown. Each column and bar represents the mean±SEM of three samples. N.S. denotes not significant. In this experiment, the lung samples from all stages were transferred onto one membrane. The kidney samples from all stages were also transferred onto a membrane. The RV and LV samples were transferred onto respective membranes. Therefore, the typical examples of Northern blot analysis shown in Fig 5 do not accurately represent the mean of multiple animals shown in this figure.
MCT rats on either day 6 or day 14, ET-1 at low doses produced an oscillation-like response only in the MCT rats on day 14, suggesting that the vascular responsiveness to ET-1 was altered at this stage. Nevertheless, the pulmonary artery exhibited a significantly reduced response to ET-1 in the MCT rats on day 25. Since the pulmonary vasoconstrictile response to prostaglandin $F_{2\alpha}$ or 5-hydroxytryptamine has been shown to be increased in MCT rats at the pulmonary hypertensive stage, the reduction in the response to ET-1 seems to be a phenomenon relatively specific for ET-1. In the present study, MCT did not affect the vasoconstrictile response to ET-1 in the aorta, indicating that the reduction in the vascular response to ET-1 occurred specifically in the pulmonary artery. ET-1 activates vascular ET$_{\alpha}$ receptors, thereby causing vasoconstriction via several intracellular signal transduction systems.

The ET$_{\alpha}$ receptor can easily be downregulated by exposure to endothelins. MCT has been reported to injure the endothelium of pulmonary arteries but not of the aorta. MCT-induced endothelial injury causes an increase in pulmonary vascular permeability and the exudation of plasma components to the pulmonary vascular wall but not to the aortic wall. Therefore, it is possible that elevated circulating ET-1 in MCT rats can easily penetrate into the pulmonary vessel wall but probably not into the aortic wall. This phenomenon may have led to the selective downregulation of ET$_{\alpha}$ receptors, thereby reducing the response to ET-1 in the pulmonary artery. Lerman et al. have reported that acute systemic administration of ET-1 producing a twofold increase in its circulating concentration results in significant cardiovascular and renal biological activity, i.e., decreased heart rate and cardiac output in association with increased renal and systemic vascular resistance in dogs. In the present study, however, systemic blood pressure did not significantly change (Table 1) despite a fivefold increase in plasma ET-1 concentra-

### Table 4. Body Weight and Wet Weight of Hearts of Control Rats and Monocrotaline-injected Rats Given Continuous Infusion of BQ-123 or Saline

<table>
<thead>
<tr>
<th>Group</th>
<th>BW, g</th>
<th>LV+SEP, mg</th>
<th>RV, mg</th>
<th>(LV+SEP)/BW, mg/g</th>
<th>RV/BW, mg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>C+Saline (n=8)</td>
<td>251.9±3.3*</td>
<td>548.0±11.5*</td>
<td>170.8±4.1*</td>
<td>2.18±0.03</td>
<td>0.68±0.011</td>
</tr>
<tr>
<td>MCT+saline (n=10)</td>
<td>227.8±5.8</td>
<td>496.5±13.7</td>
<td>279.6±20.1</td>
<td>2.18±0.05</td>
<td>1.23±0.09</td>
</tr>
<tr>
<td>MCT+BQ-123</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.1 mg per day per rat (n=9)</td>
<td>236.9±6.7</td>
<td>544.3±18.3</td>
<td>232.2±10.9*</td>
<td>2.29±0.04</td>
<td>1.01±0.05*</td>
</tr>
<tr>
<td>14.3 mg per day per rat (n=7)</td>
<td>231.9±4.1</td>
<td>534.7±21.3</td>
<td>219.7±13.0*</td>
<td>2.30±0.07</td>
<td>0.95±0.05*</td>
</tr>
</tbody>
</table>

BW indicates body weight; LV, left ventricle; SEP, septum; RV, right ventricle; C+saline, control rats given continuous saline infusion for 18 days; MCT+saline, monocrotaline (MCT)-injected rats given continuous saline infusion for 18 days; and MCT+BQ-123, MCT rats given continuous BQ-123 infusion for 18 days. Values are mean±SEM.

*P<.05 and †P<.01 vs the corresponding value for MCT+saline.

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**Fig. 8.** Photomicrographs show the effects of continuous BQ-123 infusion on the lung vascular morphology of monocrotaline (MCT)-injected rats. A, Control rats were given continuous saline infusion for 18 days. B, MCT rats were given continuous saline infusion for 18 days. C, MCT rats were given continuous BQ-123 infusion (14.3 mg per day per rat) for 18 days. Each arrow indicates the section of the pulmonary artery; Br, bronchus. Lung histology showed a marked increase in pulmonary arterial medial thickness in the MCT rats (B). Continuous infusion of BQ-123 significantly inhibited the increase in the medial thickness of the pulmonary arteries of the MCT rats (C). Bar=100 μm.
tion in MCT rats on day 18. The decrease rather than increase in systemic blood pressure on day 25 may have been due to a continuous severe sickness (see the body weight in Table 2). Therefore, it is likely that circulating ET-1 plays a relatively selective role in the alteration of the cardiopulmonary system of MCT rats. Although the exact reason for the selective reduction in the pulmonary vascular response to ET-1 is unclear, this finding suggests that excessive stimulation of ET_{A} receptors selectively occurs in the pulmonary vascular smooth muscles of the MCT rats.

In the present study, the ET_{A} receptor antagonist BQ-123 significantly inhibited the development of pulmonary hypertension and RV hypertrophy. Since ET-1 is a potent pulmonary vasoconstrictor,\textsuperscript{5,7} one possible mechanism for the inhibition of pulmonary hypertension by BQ-123 may be antagonism of the vasocontractile action of endogenous ET-1. Indeed, ET-1 induced an oscillation-like response in the pulmonary artery of the MCT rats on day 14. However, since the pulmonary vascular response to ET-1 was markedly reduced in the MCT rats on day 25, it is unlikely that the inhibition of sustained pulmonary hypertension by BQ-123 was solely due to the antagonism of ET-1-induced vasoconstriction.

Increased thickness of the pulmonary arterial media is believed to play a major role in the development of MCT-induced pulmonary hypertension and sustained irreversible increases in pulmonary vascular resistance.\textsuperscript{20,23,38} ET-1 has potent proliferative activity on vascular smooth muscle cells.\textsuperscript{4,9} The present histological examination revealed that BQ-123 effectively prevented arterial medial thickening in the MCT rats, suggesting that endogenous ET-1 participates in the thickening of pulmonary vessels. These findings are in agreement with the demonstration that ET-1-induced DNA synthesis is inhibited by BQ-123 in cultured rat vascular smooth muscle cells.\textsuperscript{39} Therefore, it is conceivable that the inhibition of pulmonary hypertension by BQ-123 is, at least in part, due to the antagonism of ET-1-induced vascular proliferation. This assumption is consistent with the above-mentioned argument that the excessive stimulation of ET_{A} receptors may selectively occur in the pulmonary vascular smooth muscle of the MCT rats.

RV hypertrophy occurred concomitantly with the development of pulmonary hypertension after MCT treatment; it was moderate on day 14 and marked after day 18. Another important finding in the present study was the dramatic increase in prepro ET-1 mRNA expression in the hearts of the MCT rats at the severe pulmonary hypertensive stage (after day 18). The increase in prepro ET-1 mRNA expression in the heart may have been due to overload of the heart caused by severe pulmonary hypertension. It has been demonstrated that the 5' flanking region of the prepro ET-1 gene has three octanucleotide sequences that conform with a consensus of AP-1/Jun–binding elements and that phorbol esters actually activate prepro ET-1 mRNA expression in cultured endothelial cells.\textsuperscript{40} Furthermore, pressure overload to the heart has been shown to cause an increase in c-fos and c-jun expression in the early stage.\textsuperscript{41} Therefore, it is likely that pressure overload to the heart may induce prepro ET-1 mRNA expression via the expression of the trans-acting transcription factors Fos and Jun. Since ET-1 was shown to induce myocardial cell hypertrophy,\textsuperscript{16} the marked increase in ET-1 production in the heart may also contribute to an exaggeration of cardiac hypertrophy after day 18. We believe that the inhibition of RV hypertrophy by BQ-123 can be partly attributed to the blockade of excessive stimulation of the heart by ET-1, in addition to the prevention of pulmonary hypertension.

It has been reported that lung ET-1 production increased in rats with idiopathic pulmonary hypertension despite a lack of increase in plasma ET-1.\textsuperscript{42} In contrast, lung prepro ET-1 mRNA expression in rats with pulmonary hypertension due to chronic hypoxia does not differ from that in control rats.\textsuperscript{42} Thus, lung ET-1 production appears to differ among animal models of pulmonary hypertension. In the present study, lung ET-1 production decreased in the MCT rats. As a possible mechanism for this, it can be speculated that severe inflammation of the lung parenchyma or severe damage of the pulmonary endothelial cells caused by
MCT might result in a reduction of ET-1 production in the lung. The presence of injured vascular endothelial cells in the pulmonary circulation, but not in the systemic circulation, has been shown in patients with pulmonary hypertension. Furthermore, increased circulating levels of ET-1 have been demonstrated in patients with both primary and secondary forms of pulmonary hypertension. These phenomena are similar to those seen in the MCT rats.

It has been reported that platelet-activating factor antagonists inhibit MCT-induced lung injury and pulmonary hypertension. In addition, p-chloroamphetamine, a 5-hydroxytryptamine synthesis inhibitor, has also been shown to inhibit MCT-induced pulmonary hypertension in rats. Thus, MCT-induced pulmonary hypertension appears to be caused by several etiologic factors. In the present study, we demonstrated that an ET\(_4\) receptor antagonist inhibited the development of pulmonary hypertension, suggesting that endogenous ET-1 probably contributes to the progression of cardiopulmonary alterations in MCT rats. Although the precise mechanism of the inhibitory effects of an ET\(_4\) receptor antagonist is unclear, the present findings may provide new directions for analyzing the mechanisms of progression of pulmonary hypertension and for developing beneficial treatments for pulmonary hypertension.

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Contribution of endogenous endothelin-1 to the progression of cardiopulmonary alterations in rats with monocrotaline-induced pulmonary hypertension.

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