Alterations of endothelium and smooth muscle function in monocrotaline-induced pulmonary hypertensive arteries

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Alterations of endothelium and smooth muscle function in monocrotaline-induced pulmonary hypertensive arteries. Am J Physiol Heart Circ Physiol 279: H1786–H1795, 2000.—We examined how monocrotaline (MCT), which impairs the endothelium and causes pulmonary hypertension, altered the endothelial regulation of pulmonary artery functions. Rats were given a single injection of MCT (60 mg/kg sc). Pulmonary arteries were depolarized to $-48.3 \pm 2.6$ and $-39.8 \pm 2.2$ mV at 2 and 3 wk after treatment with MCT, respectively (control arteries $-59.9 \pm 1.9$ mV). The basal tone in the resting state was only slightly elevated at 3 wk in endothelium-intact arteries. Removal of the endothelium caused further depolarization in MCT-affected arteries at 2 wk, but not at 3 wk, and greatly elevated the basal tone at 2 and 3 wk. $N^\omega$-nitro-L-arginine (200 $\mu$M), a nitric oxide synthase inhibitor, also caused depolarization in endothelium-intact arteries in both groups and elevated the basal tone of MCT-affected arteries. The relaxant responses of pulmonary arteries to ACh and A-23187 were depressed at 2 and 3 wk after MCT treatment. Thus chronic impairment of the endothelium altered the property of the pulmonary artery leading to depolarization. During the early stage of depolarization, a rise in the basal tone was offset by nitric oxide released from the injured endothelium.

nitric oxide; membrane potential; depolarization; sodium nitroprusside; resting tone

MONOCROTALINE (MCT) is an inactive alkaloid obtained from seeds of Crotalaria sp. and is biotransformed to toxic metabolites such as MCT pyrrole in the liver (12). When MCT is administered systemically (5, 9, 17), the arterial bed that is first encountered by the metabolite is the pulmonary circulation, so it preferentially injures the pulmonary artery endothelium (12, 36). As a result of the impairment, an animal given MCT exhibits pulmonary hypertension characterized by an increase in pulmonary artery resistance and right ventricular hypertrophy (5, 9, 17, 30). For this characteristic, this alkaloid has been used as a model to explore the process of pulmonary hypertension or the resultant cardiac hypertrophy and to find an appropriate intervention for treatment of pulmonary hypertension-related diseases. To clarify why the vascular resistance increases during MCT-induced pulmonary hypertension, several attempts have been made by observing responses of MCT-affected pulmonary arteries to vasorelaxing or vasoconstricting agents. However, the data from such studies were conflicting, i.e., responses to vasoconstricting agents, which varied from enhancement (10, 11) to depression (2, 15, 32), compared with the control responses. This inconsistency means that alterations of responsiveness to vasodilating substances do not have a causal relationship to an increase in the pulmonary artery resistance.

The resting membrane potential is an important determinant of vascular tone. The endothelium exerts a hyperpolarizing effect on the underlying vascular smooth muscle through the action of nitric oxide (NO) (29), prostacyclin (20), or endothelium-derived hyperpolarizing factor (EDHF) liberated from the endothelium (21) or through direct coupling between endothelial cells and smooth muscle cells (35). In 1982, Suzuki and Twarog (27) showed that the main pulmonary artery was depolarized, while the small pulmonary artery was hyperpolarized, during MCT- or hypoxia-induced pulmonary hypertension in rats. However, at the time of their work, the importance of endothelial control on the membrane property of smooth muscle cells had not been recognized, and it is uncertain whether the endothelium was preserved in their preparations. Since that time, the relationship between the endothelium and electrical properties of vascular smooth muscle in this model has not been thoroughly investigated. Therefore, it is still unclear whether an increase in vascular resistance is associated with a change in the membrane potential in this model. In this study we observed morphological and functional changes in the pulmonary artery in rats treated with MCT and examined how the hyperpolarizing and relaxing effects of the endothelium and the membrane property of vascular smooth muscle cells were altered during the MCT-induced pulmonary hypertension.

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MATERIALS AND METHODS

Preparations. Six-week-old male Wistar rats received a single injection of MCT (60 mg/kg sc; Sigma Chemical, St. Louis, MO), which had been dissolved in 1 N HCl, diluted into saline, and neutralized to pH 7.4 with NaOH, or the same volume of saline (control). The rats were fed and given water ad libitum. At 7, 14, or 21 days after the injection, the rats were killed under light ether anesthesia. The lung, the right ventricle, and the left ventricle attached to the septum were isolated from rats used for the tension experiments, and their weights were measured. The main pulmonary artery was dissected, and connective tissues were cleaned off. The artery segment was cut open, and a 5-mm-long, 2-mm-wide strip was made. When needed, the endothelium was gently removed with a cotton swab.

Morphological study. For light-microscopic examination of the artery, isolated main pulmonary artery rings were fixed in 10% Formalin and embedded in paraffin (immersion fixation). The sections were sliced at 4 μm and stained with hematoxylin-eosin or resorcin-fuchsin. The latter staining was used to specify the locus of the innermost lamina in the medial layer. In a separate experiment to observe the endothelium in a condition closer to that in vivo, rats were anesthetized with pentobarbital sodium (50 mg/kg), and the caudal vena cava was cannulated with a polyethylene tube. Physiological saline solution (PSS) was infused from the cannula and drained from an incision in the abdominal aorta, and 10% Formalin was slowly infused (perfusion fixation). A segment of the main pulmonary artery was then isolated and processed for light microscopy as described above. A microscopic image of the cross section of artery was acquired by a computer. To measure the distance between the endothelial cell and the medial layer, the inner circumference of the artery ring was marked at three points with an equal distance. The distance was measured at each point by use of Canvas 6.0 software and averaged.

To measure the cross-sectional area of the artery, the muscle strips that had been used for tension experiments were sandwiched in an incision made in a rat liver block, fixed with 10% Formalin, and processed for light microscopy. This procedure prohibited a collapse of thin strips during tissue preparation and allowed precise measurement for normalization of the muscle tension to a cross-sectional area of artery. A cross-sectional area of the medial layer was measured using Canvas 6.0 after the image had been acquired by a computer.

Tension measurement. The strip was suspended in an organ bath containing 10 ml of PSS of the following composition (mM): 116.8 NaCl, 5.4 KCl, 2.5 CaCl₂, 1.0 MgCl₂, 11.9 NaHCO₃, and 5.5 glucose (pH 7.3–7.4 when gassed with 95% O₂-5% CO₂) at 37°C. A change in tension was recorded isometrically under resting tension of 400 mg. To measure the resting tension developed spontaneously under the resting condition (basal tone), preparations were stabilized by three challenges with high-KCl solution, and the external medium was then switched from PSS to Ca²⁺-free PSS containing 1 mM EGTA. A drop of the tension from the resting level was measured. A high-KCl (65.4 mM) solution was made by replacement of 60 mM NaCl for isosmolar KC1. When the endothelium-dependent relaxation was measured, the artery was precontracted by 10 μM PGF₂α, and ACh or A-23187 was then cumulatively added.

Membrane potential measurement. The strip was mounted horizontally in a superfusion bath with the adventitial layer facing up. The preparation was superfused with PSS at a rate of 4 ml/min. A glass microelectrode with a tip resistance of 60–80 MΩ when filled with 3 M KCl was penetrated into a cell from the adventitial side. The signal was fed into a microelectrode amplifier (model MEZ-8301, Nihon-Kohden, Tokyo, Japan) and recorded on a pen-writing oscillograph (San’eï Recti-Hori, Tokyo, Japan) or stored on a VCR tape by use of a modified PCM processor (model PCM-501 ES, Sony, Tokyo, Japan).

Cytosolic Ca²⁺ concentration measurement. For measurement of the cytosolic Ca²⁺ concentration ([Ca²⁺]i) of the pulmonary artery, the strip was loaded overnight with 5 μM fura PE3-AM (TEFLAB, Austin, TX) that had been sonicated together with 0.02% cremophore EL in PSS. Overnight loading did not affect the contractile response to 65.4 mM KCl or PGF₂α. The strip was then placed in a bath constructed in a fluorometer (model CAF-100, JASCO, Tokyo, Japan). Fluorescence at 500 nm after excitation at 340 and 380 nm was monitored. At the end of the experiment, 10 μM ionomycin and then 10 mM EGTA were applied to obtain fluorescence at maximum and minimum [Ca²⁺]i, respectively. The signals of the tension and [Ca²⁺]i were stored on the hard disk of an IBM-compatible personal computer, and the data were analyzed with Microsoft Excel. Background fluorescence was subtracted from all the data. [Ca²⁺]i was calculated as described by Grynkiewicz et al. (8) with use of a dissociation constant for fura PE3 of 290 nM (31).

Values are means ± SE. Student’s t-test was performed for comparison of two values. For multiple comparison, ANOVA was followed by post hoc test (Bonferroni/Dunn method). Statistical significance was considered at P < 0.05.

RESULTS

Development of pulmonary hypertension. After injection of MCT (60 mg/kg sc), the increase in body weight was less at 3 wk than in the control group (data not shown). The lung-to-body weight ratio increased at 2 and 3 wk after the injection of MCT, whereas that in the control group decreased at later times. The right ventricle-to-left ventricle + septum weight ratio was larger at 2 and 3 wk in the MCT group than in the control group (Table 1). Because these changes have been considered an index of pulmonary hypertension (5, 17), it is suggested that the pulmonary hypertension and the consequent right ventricular hypertrophy were established between 2 and 3 wk after MCT treatment in this study. At 4 wk after the injection, about one-half of the animals treated with MCT died. Therefore, we did not use the pulmonary artery after 4 wk.

Table 1. Lung-to-body weight ratio and right ventricle-to-left ventricle + septum weight ratio in rats treated with MCT or saline

<table>
<thead>
<tr>
<th>Time After Treatment</th>
<th>1 wk</th>
<th>2 wk</th>
<th>3 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung-to-body wt ratio</td>
<td>Control 0.675 ± 0.048 (14) 0.566 ± 0.027 (18) 0.551 ± 0.025 (17)</td>
<td>MCT 0.761 ± 0.026 (16) 0.784 ± 0.029* (21) 0.880 ± 0.040* (16)</td>
<td></td>
</tr>
<tr>
<td>Right ventricle-to-left ventricle + septum wt ratio</td>
<td>Control 0.256 ± 0.032 (14) 0.239 ± 0.042 (18) 0.284 ± 0.011 (17)</td>
<td>MCT 0.276 ± 0.045 (16) 0.327 ± 0.020* (21) 0.384 ± 0.028* (16)</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE of number of preparations in parentheses. MCT, monocrotaline. *P < 0.05 vs. time-matched control.
Microscopic examination of the pulmonary artery, which was fixed by immersion in Formalin, revealed medial thickening and detachment of the endothelial layer from the medial layer in the main pulmonary artery at 2 or 3 wk after treatment with MCT (Fig. 1, Table 2). Because we thought that the endothelium might have become detached from the artery during processing for fixation, we reexamined the distance in perfusion-fixed arteries, the data from which are supposed to more closely reflect the actual distance in vivo than data from immersion-fixed arteries. The distance in the perfusion-fixed arteries was similar to that in the immersion-fixed arteries at 2 wk but less at 3 wk (Table 2). The overall data suggest that the endothelial cells of MCT-affected arteries were loosely connected to the smooth muscle layer and were going to easily exfoliate at later times. In the inner part of medial layer, the nuclei of some smooth muscle cells were oriented with their longer axes toward a center of vessel lumen. We observed no migration of smooth muscle cells into the intima.

In a preparation where endothelial cells were detached from the inner lamina, monocytes or polymorphonuclear cells sometimes adhered beneath the endothelium (Fig. 1). Thickening of the adventitial layer was also observed at 2 or 3 wk after treatment with MCT, although we did not accurately measure it.

### Table 2. Cross-sectional area of medial layer and detachment of endothelial layer from inner lamina of pulmonary arteries from rats treated with MCT or saline

<table>
<thead>
<tr>
<th>Time After Treatment</th>
<th>Control</th>
<th>MCT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cross-sectional area of medial layer, mm²</td>
<td></td>
</tr>
<tr>
<td>Control 1 wk</td>
<td>0.72 ± 0.10</td>
<td>0.79 ± 0.08</td>
</tr>
<tr>
<td>Control 2 wk</td>
<td>0.58 ± 0.08</td>
<td>1.20 ± 0.07</td>
</tr>
<tr>
<td>Control 3 wk</td>
<td>0.83 ± 0.06</td>
<td>1.14 ± 0.15</td>
</tr>
<tr>
<td>MCT 1 wk</td>
<td>0.79 ± 0.08</td>
<td>1.20 ± 0.07</td>
</tr>
<tr>
<td>MCT 2 wk</td>
<td>1.14 ± 0.15</td>
<td>1.20 ± 0.07</td>
</tr>
<tr>
<td>MCT 3 wk</td>
<td>1.54 ± 0.20</td>
<td>1.20 ± 0.07</td>
</tr>
</tbody>
</table>

Detachment of endothelial layer, μm:

<table>
<thead>
<tr>
<th>Time After Treatment</th>
<th>Control</th>
<th>MCT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Immersion fixation</td>
<td>Perfusion fixation</td>
</tr>
<tr>
<td>Control 1 wk</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>Control 2 wk</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Control 3 wk</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MCT 1 wk</td>
<td>0.87 ± 0.37</td>
<td>5.89 ± 0.52</td>
</tr>
<tr>
<td>MCT 2 wk</td>
<td>5.20 ± 1.89</td>
<td>6.48 ± 0.31</td>
</tr>
<tr>
<td>MCT 3 wk</td>
<td>12.30 ± 1.56</td>
<td>6.48 ± 0.31</td>
</tr>
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</table>

Values are means ± SE of 7–14 preparations. Detachment was measured in preparations processed by immersion fixation or perfusion fixation. ND, not determined. *P < 0.05 vs. time-matched control.

**Development of the basal tone and decrease in the resting membrane potential.** After each strip was equilibrated with PSS in an organ bath under basal tension of 400 mg, the external medium was switched to Ca²⁺-free PSS containing 1 mM EGTA. In a preparation where the endothelium was preserved, Ca²⁺-free EGTA solution did not lower the tension in control arteries and in those treated with MCT for 1 or 2 wk. However, it slightly lowered the tension in the arteries at 3 wk after treatment with MCT, suggesting that the MCT-affected arteries at this stage had an active basal tone in the resting state (Fig. 2, A and B). When the endothelium was removed, the basal tone was increased in the control and MCT-affected arteries. The basal tone of endothelium-denuded arteries after 2 or 3 wk of treatment with MCT was much greater than that in the time-matched controls (Fig. 2B). In a preliminary experiment, we stretched the endothelium-intact artery to 1.3 times the original length instead of subjecting it to 0.4 g of pretension and measured the basal tension by changing the external medium to Ca²⁺-free EGTA solution. In this case, too, the basal tension was higher in arteries at 3 wk after treatment with MCT than in control arteries (data not shown). Therefore, it is obvious that the tension in the resting state increased in MCT-affected arteries irrespective of the experimental condition to give pretension.

At 1 wk after MCT treatment, the resting membrane potential of intact pulmonary arteries (−58.4 ± 1.5 mV, n = 16) was not significantly different from that of the control group (−59.9 ± 1.9 mV, n = 14). At 2 or 3 wk after MCT, the resting membrane potential decreased progressively, whereas that in the control group remained constant (Fig. 2C). Spontaneous spike activity was observed in arteries at 2 wk and, more frequently, 3 wk after treatment with MCT (see Fig. 5).
When the endothelium was removed from the control arteries, the resting membrane potential was depolarized by 5.6 ± 0.6 mV (n = 14). Removal of the endothelium also depolarized the arteries at 2 wk after treatment with MCT and increased the amplitude of spike, indicating that the spike activity depended on the membrane potential. The magnitude of depolarization on removal of endothelium in that group (6.5 ± 1.3 mV, n = 9) was similar to that in the control group. However, the deendothelialization did not significantly enhance depolarization of the arteries at 3 wk after treatment with MCT (Fig. 2).

As well as the endothelial denudation, addition of Nω-nitro-L-arginine (L-NNA, 200 μM), an NO synthase inhibitor, significantly depolarized the membrane potential in control and MCT-affected arteries in which the endothelium was preserved (Fig. 3A). The magnitude of depolarization due to L-NNA in control and MCT-affected arteries at 3 wk after treatment was similar (6.3 ± 2.0 and 6.1 ± 1.9 mV, n = 6, respectively), despite different resting membrane potentials. L-NNA increased the basal tone in MCT-affected arteries but not in control arteries (Fig. 3B).

Response to NO-dependent vasodilators. Figure 4 shows the dose-dependent relaxant response to ACh and A-23187 in control and MCT-affected arteries precontracted with 10 μM PGF2α. The developed tension per cross-sectional area of the medial layer in response to 10 μM PGF2α in the MCT group at each week was not different from that in the time-matched control. The relaxation to ACh in arteries at 1 wk after treatment with MCT was not different from the control condition but was significantly depressed at 2 and 3 wk. The depression of the response was maximum at 2 wk after treatment. The relaxant response to A-23187 at the lowest concentration was depressed at 1 wk after treatment with MCT, and the overall responses were depressed at 2 or 3 wk. ACh- and A-23187-induced relaxations were abolished by endothelial denudation or pretreatment with 200 μM L-NNA.
ACh (1 μM) caused hyperpolarization in control and MCT-affected arteries (Fig. 5). Accompanied with the hyperpolarization, spontaneous spike activity in MCT-affected arteries was decreased or ceased. The magnitude of ACh-induced hyperpolarization in MCT-affected arteries was not different from that in control arteries (Fig. 5B). ACh-induced hyperpolarization was abolished in both groups when the arteries were pre-treated with 200 μM L-NNA or when the endothelium had been denuded.

On the other hand, 1 μM sodium nitroprusside (SNP), an NO donor, also hyperpolarized the membrane in both groups at 3 wk after treatment (Fig. 6). The SNP-induced hyperpolarization did not depend on the presence of endothelium but, rather, on the membrane potential, such that the hyperpolarization was larger when the resting membrane potential was more positive (Fig. 6B).

Changes in [Ca^{2+}]_i during washout with Ca^{2+}-free medium. As shown in Fig. 2A, when a contractile stimulation (high KCl) was removed, relaxation of MCT-affected arteries was slow compared with that of control arteries. This was also observed in the case of PGF_{2α} -induced contraction (data not shown). To observe whether the depression of relaxation accompanied a slow decline of [Ca^{2+}]_i, we measured [Ca^{2+}]_i in fura PE3-AM-loaded strips. The deendothelialized artery was contracted by 65.4 mM KCl, and 5 min later it was rinsed with Ca^{2+}-free 1 mM EGTA solution.

Fig. 3. Effects of 200 μM Nω-nitro-L-arginine (L-NNA) on membrane potential and basal tone of the endothelium-intact pulmonary artery. A: example record of effect of L-NNA in arteries at 3 wk after treatment with MCT or saline. B: summarized data of changes in basal tone of arteries at 2 or 3 wk after treatment with MCT or saline. Values are means ± SE of 8 preparations. *P < 0.05 vs. control.

Fig. 4. Dose-response relationship for ACh- or A-23187-induced relaxation of pulmonary arteries 1, 2, and 3 wk after injection of MCT (●) or saline (○). After endothelium-intact arteries were contracted with 10 μM PGF_{2α} for 10 min, ACh or A-23187 was cumulatively added. Values are means ± SE of 7–9 preparations. *P < 0.05 vs. control.
At the maximum contraction due to KCl, $[Ca^{2+}]_i$ and tension, expressed as an absolute value, were significantly higher in arteries at 3 wk after treatment with MCT than in control arteries, although the tension per cross-sectional area was not different between the two groups (387 ± 46 and 407 ± 75 mg/mm² in control and MCT groups, respectively). During rinsing with Ca²⁺-free EGTA solution, $[Ca^{2+}]_i$ and tension were always higher in the MCT-treated group than in the control group (Fig. 7, A and C). To compare the rate of decline of $[Ca^{2+}]_i$ and tension, the data were normalized to the respective peak value in each preparation. For kinetics analysis, $[Ca^{2+}]_i$ and tension are expressed on a logarithmic scale (Fig. 7, B and D). Obviously, the rate of decline in $[Ca^{2+}]_i$ or tension was slowed in MCT-affected arteries. The $[Ca^{2+}]_i$ curve is a sum of two exponential decays. The rate constant for the early phase was 0.96 and 0.88 min⁻¹ in control and MCT groups (half-time = 0.72 and 0.79 min), respectively, whereas that for the late phase was 0.16 and 0.13 min⁻¹ (half-time = 4.3 and 5.2 min), respectively.

DISCUSSION

From the results in the present study, temporal changes in structures and functions of the pulmonary endothelium and vascular smooth muscle after treatment with MCT are summarized as follows: 1) Thickening of the medial layer and depression of the relaxant response to ACh or A-23187 were evident at 2 wk after MCT treatment and exhibited no further development at 3 wk. 2) Detachment of endothelial cells and depolarization were more severe at 3 wk than at 2 wk. Also, the lung-to-body weight ratio and the right ventricle-to-left ventricle + septum weight ratio were also higher at 3 wk than at 2 wk. 3) Elevation of basal tone in the resting state of endothelium-intact pulmonary arteries was observed only at 3 wk after treatment with MCT, whereas that of deendothelialized arteries was evident at 2 wk and, more intensely, at 3 wk. Temporal dissociation among the above parameters suggests that a primary toxic effect of MCT on the endothelium, which was typically shown as depression of the endothelium-dependent relaxant response, was maximal at 2 wk, whereas some of changes secondary
to endothelial impairment (depolarization, an increase in the basal tone, and ventricular hypertrophy) were progressive and aggravate the pathological condition, which became lethal beyond 3 wk.

The pulmonary endothelium exerted a hyperpolarizing effect on smooth muscle cells in the resting state in control and MCT-affected arteries, since removal of the endothelium or addition of L-NNA caused depolarization of the smooth muscle. ACh-induced hyperpolarization was endothelium dependent and was blocked by pretreatment with L-NNA. The NO donor SNP hyperpolarized the membrane, consistent with the reports that NO hyperpolarized the rat pulmonary artery (3, 37). Taken together, these facts indicate that NO mediates the endothelium-dependent hyperpolarization in rat pulmonary arteries in the resting state or after stimulation with ACh. An EDHF, which is not a product of NO synthase or cyclooxygenase (21), is unlikely to exert a significant hyperpolarizing effect in the preparation tested here.

The hyperpolarization response to SNP was larger when the resting membrane potential was more depolarized. This is reasonable, because NO-induced hyperpolarization is due to activation of K⁺ channels (19, 24, 37), and the hyperpolarization as a result of K⁺ channel activation is larger when the membrane potential is more positive to the equilibrium potential for K⁺. In contrast to the response to SNP, the hyperpolarization to ACh was similar in MCT-affected and control arteries. Likewise, the depolarization caused by removal of endothelium or addition of L-NNA was also similar in the two groups. If the amount of NO available to smooth muscle cells is the same, the resultant hyperpolarization should be larger in more depolarized arteries (i.e., MCT-affected arteries). Compared with the response to SNP, the response to ACh, L-NNA, or deendothelialization was too small in MCT-affected arteries. Consequently, we conclude that the amount of NO that reached smooth muscle cells was less in MCT-affected than in control arteries when the arteries were

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**Fig. 6.** Hyperpolarizing effects of 1 μM sodium nitroprusside (SNP) on resting membrane potential of pulmonary arteries after 3 wk of treatment with MCT or saline (control). 

A: example records of the effect of SNP in endothelium-intact or -denuded arteries. 

B: relationship between resting membrane potential and the response to SNP. Open symbols, control arteries; filled symbols, MCT-affected arteries; circles, response to SNP in endothelium-intact arteries; squares, response to SNP in endothelium-denuded arteries. Values are means ± SE of 7 (control) and 8 experiments (MCT). SNP significantly hyperpolarized the membrane in both groups.
rested or when they were stimulated with ACh. Depression of the endothelium-dependent relaxant response to ACh or A-23187 after treatment with MCT supports this idea.

However, this does not necessarily mean that NO production in endothelial cells was reduced after treatment with MCT. It has been shown that expression of endothelial NO synthase was increased rather than decreased in cultured endothelial cells treated with MCT (26) and that NO production in the MCT-injured lung was similar to that in the control lung (34). Consistent with these findings, mRNA encoding endothelial NO synthase was increased in MCT-affected pulmonary arteries (22). From these reports, it is conceivable that the MCT-affected endothelium is still capable of producing NO in an amount comparable to that produced by the control endothelium. Although we did not measure production of NO, even if the synthesis of NO was not decreased, it is possible that a quantity of NO molecules released from endothelial cells diffused away from smooth muscle cells of MCT-affected arteries, because endothelial cells exfoliated from the smooth muscle cells. It is also possible that if depolarization occurs in MCT-injured endothelial cells, a decrease in electrochemical Ca\(^{2+}\) gradient would attenuate Ca\(^{2+}\) entry into endothelial cells and thereby reduce production of NO (13).

Removal of the endothelium depolarized control arteries by \(-6\) mV. In contrast, the resting membrane potential of MCT-affected arteries was \(10–20\) mV more positive than that of control arteries. Therefore, a decrease in the hyperpolarizing effect of the endothelium is not a sole mechanism for the depolarization of MCT-affected arteries. Obviously, the membrane property of smooth muscle cells was altered toward depolarization after treatment with MCT. Because MCT pyrrole, an active metabolite of MCT (12, 36), did not exert any influence on cultured pulmonary artery cells, whereas it damaged endothelial cells (25), the alteration in the membrane property was probably secondary to long-
term impairment of the endothelium but not a result of the direct action of the compound on smooth muscle cells. A problem regarding changes in smooth muscle functions is why a long-term decrease of endothelial control alters the membrane property of smooth muscle cells. It is possible that when the normal endothelium-smooth muscle coupling is disrupted, some factor from leukocytes, platelets, or endothelial cells is released (6, 7) and alters the properties of endothelial and smooth muscle cells. Alternatively, under control of these factors, proliferated smooth muscle cells could show a phenotype different from normal cells. Adhesion of leukocytes to endothelial cells in MCT-affected arteries suggests that endothelium-leukocyte interaction affected smooth muscle cells (14, 23). It remains to be clarified what factor(s) is responsible for the remodeling of the pulmonary artery.

An interesting finding in this study is that, despite considerable depolarization, the basal tone did not develop (1–2 wk) or only slightly developed (3 wk) in MCT-affected arteries when the endothelium was preserved. This suggests that the amount of NO released from impaired endothelial cells in the resting state was enough to cancel the influence of depolarization on the resting tension. Only when the membrane potential is depolarized to around −40 mV or spontaneous release of NO decreases considerably, the tone in the resting state may increase. At 2 wk after treatment with MCT, right ventricular hypertrophy already appeared, whereas tonus of main pulmonary artery (endothelium intact) did not develop. This yields the impression that a change in the artery is not responsible for development of pulmonary hypertension and ventricular hypertrophy. In our preliminary experiments, however, the basal tone of the intrapulmonary artery was already elevated at 2 wk (unpublished data), suggesting that the impairment is likely to occur earlier in smaller arteries. Narrowing of the vessel lumen as a result of medial thickening and increase in the tonus of smaller arteries may contribute to an increase in the pulmonary artery resistance at an earlier stage, leading to right ventricular hypertrophy. Beyond 3 wk, however, the basal tone would be elevated in large arteries; this could worsen the condition.

Na⁺/Ca²⁺ exchange is one of determinants of [Ca²⁺] i. Na⁺/Ca²⁺ exchange in smooth muscle is eletrogenic in nature (1, 16); therefore, it depends on the membrane potential. After the high-K⁺ medium was switched to Ca²⁺-free EGTA solution (5.4 mM K⁺, [Ca²⁺] i), decreased with kinetics of double-exponential decay. When high K⁺ is removed, the membrane potential cannot rapidly repolarize to the resting level. Because Ca²⁺ extrusion through the exchanger occurs at a voltage negative to the equilibration potential for Na⁺/Ca²⁺ exchange (18), Ca²⁺ extrusion through Na⁺/Ca²⁺ exchange may be reflected in the late component of [Ca²⁺] i decay. Therefore, a smaller rate constant of the late component in the MCT group means that the depolarization inhibited the Ca²⁺ extrusion through the Na⁺/Ca²⁺ exchange. This change in Ca²⁺ handling increases [Ca²⁺] i at the steady state and contributes to an increase in the basal tone. A deficiency in Ca²⁺ extrusion through Na⁺/Ca²⁺ exchange may be less as long as the endothelium releases NO, since a GMP-elevating agent stimulates Na⁺/Ca²⁺ exchange (4). Consistent with this, an increase in the basal tone was quite small in endothelium-intact arteries in the MCT-affected group. Another contribution of depolarization to an increase in the basal tone is activation of L-type Ca²⁺ channels. This appears to occur in MCT-induced hypertensive vessels, since Ca²⁺ channel blockers more effectively inhibited the norepinephrine- or serotonin-induced contractions in MCT-affected than in control arteries (28, 33).

In summary, as a result of damage to the endothelium by MCT, the influence of the endothelium on smooth muscle cells is reduced and the membrane property of smooth muscle cells is altered, leading to depolarization of the pulmonary artery. At the early stage of depolarization, however, tension of the pulmonary artery is kept low by the relaxing action of NO released from the injured endothelium.

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