Effect of Endothelium of Pulmonary Artery Vasoreactivity in Monocrotaline-Induced Pulmonary Hypertensive Rats

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This study investigated vasoreactivity modulated by endothelium in pulmonary arteries from isolated monocrotaline (MCT)-induced pulmonary hypertensive rats. The responses to KCl, 5-hydroxytryptamine (5-HT), acetylcholine (ACh), and nitroglycerin (NTG) were studied in the pulmonary artery with and without endothelium for 3 weeks following MCT-treatment. The sensitivity of the contractile response to KCl markedly increased in the arteries with endothelium at 2 and 3 weeks after treatment, but only slightly in the arteries without endothelium. In addition, the sensitivity of the contractile response to 5-HT peaked 2 weeks after treatment in the arteries with endothelium, but at 1 week in those without endothelium. Although the removal of endothelium shifted the concentration-response curve for KCl and 5-HT to the left both in the control state and at 1 week, it did not shift the curves at 2 or 3 weeks. The relaxation responses to ACh and NTG, indicative of endothelium-dependent and -independent relaxation, respectively, as well as the content of tissue cGMP were reduced 2 weeks after treatment. These results suggest that the impairment of both the endothelium-dependent and -independent relaxation responses contributes to hyperreactivity of the pulmonary artery, and may in part contribute to the development of MCT-induced pulmonary hypertension.

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MONOCROTALINE (MCT) is a toxic pyrrolizidine alkaloid extracted from the plant *Crotalaria spectabilis*. A single administration of MCT or its active metabolite, monocrotaline pyrrole (MCTP), can induce pulmonary edema,¹ pulmonary arteritis,² pulmonary hypertension, and right ventricular hypertrophy^{3,4} in rats. MCT- and MCTP-treated rats have been widely used as experimental models for primary pulmonary hypertension. Pathological studies of these models have shown endothelial injury occurring 4 days after MCT-treatment⁵ and exfoliation of many endothelial cells after 4

weeks.⁶ Increases in interstitial cells⁷ and medial hypertrophy^{8–10} have been reported in vascular smooth muscle. There are several studies of functional changes in the pulmonary arteries of MCT-treated rats. In our laboratory, Kataoka and Tobise reported hyperreactivity to KCl, CaCl₂, and 5-hydroxytryptamine (5-HT) in pulmonary arteries with endothelium.¹¹ Other studies reported that maximum contractility to vasoconstrictive agents, KCl, angiotensin II, norepinephrine, 5-HT, and endothelin was reduced in pulmonary arteries in the advanced stages of this model.^{12–14} In addition, several studies have found that the relaxation responses to acetylcholine (ACh)^{12,15} and sodium nitroprusside¹⁶ are reduced with the development of pulmonary

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hypertension.

Furchgott and Zawadzki first suggested that endothelial cells stimulated by acetylcholine released an endothelium-derived relaxing factor (EDRF).¹⁷ The endothelium attenuates the sensitivity of the contractile response to some vasoconstrictive agents through the EDRF/NO or other factors.¹⁸ Vasoreactivity may be modulated by endothelium in the pulmonary arteries of MCT-treated rats. However, less is known about the role of this vasoreactivity with regard to the pathogenesis of MCT-induced pulmonary hypertension; the present study examines this possible connection.

METHODS

One hundred male Sprague-Dawley rats (Charles River Japan Inc, Japan), 5 weeks old at the start of treatment, were divided into 2 groups (MCT group: 52; control group: 48). One group was given a single subcutaneous injection of MCT in a concentration of 40 mg/kg (Crotaline; Sigma Chemical Co, St Louis, Missouri); the control group was given a comparable volume of saline solution. No rats died in the first 2 weeks following MCT treatment. However, the mortality rate in the 3 weeks was 20.0%. At 1, 2, and 3 weeks after MCT treatment, groups of rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (40 mg/kg) and ventilated with room air using a Shinano SN-480-6 respirator (tidal volume, 10 ml/kg; respiratory rate, 90/min). We inserted an 18G needle directly into the right ventricle through the free wall. After measuring right ventricular systolic pressure with a pressure transducer (MPU-0.5, Nihon Koden Inc, Japan), we excised the heart and lungs, and immediately immersed them in Krebs-Henseleit buffer (KHB) at room temperature. The left main pulmonary artery was then isolated. In one group, without endothelium, the arterial endothelium was removed using a steel needle; in the other group, with endothelium, the arterial inner walls were left intact. Each artery was then cut into 3 mm long cylindrical segments. The vessels were suspended between two L-shaped stainless-steel hooks in an organ bath containing 50 ml of KHB, whose composition was as follows (in

mmol/L): NaCl, 118.1; KCl, 4.7; CaCl₂, 2.5; KH₂PO₄, 1.2; MgSO₄, 1.2; NaHCO₃, 25.0; and glucose, 11.0. The buffer was constantly bubbled with a 95% O₂-5% CO₂ gas mixture and maintained at 37±0.1 °C.

The arterial segment was suspended for 2 h at 1 g of applied tension. The functional status of the endothelium was ascertained by measuring the relaxant effects of ACh after precontraction with Phe (5×10⁻⁷ mol/L). The cumulative concentration-response curves were generated by KCl (8–100 mmol/L), and 5-HT (10⁻⁸–5×10⁻⁴ mol/L). The cumulative concentration-response curves were generated by ACh (10⁻⁹–10⁻⁵ mol/L) and nitroglycerin (NTG) (10⁻⁸–10⁻⁵ mol/L) after the arteries produced a tonic contraction induced by Phe (5×10⁻⁷ mol/L). The changes in isometric tension were measured using force displacement transducers (FD pick up, TB 612 T; Nihon Koden Inc, Japan) with an X-Y recorder (D 72 BP; Riken Densi, Japan). The responses to KCl and 5-HT were represented as percentages of maximum contraction to each agonist, and responses to ACh and NTG were represented as percentages of contraction to Phe (5×10⁻⁷ mol/L). The right heart ventricle (RV) was separated from the left ventricle and interventricular septum (LV+IVS). The weight ratio of RV/(LV+IVS) was used as an index of right ventricular hypertrophy. To determine the production of endothelium-derived relaxing factor (EDRF), the content of tissue cGMP was measured at resting tension (1 g), 5 min after stimulation by ACh (10⁻⁵) or after contraction at 100 mg of applied tension by Phe.

Results were expressed as mean±SEM. The concentration-response curve to each agonist was statistically analyzed by two-way analysis of variance (ANOVA). The content of tissue cGMP was statistically analyzed by one-way ANOVA, and the other data were assessed by unpaired t-test. The level of significance was $p < 0.05$.

RESULTS

Body Weight, Right Ventricular Systolic Pressure and Ventricular Heart Weight

The body weight of MCT-treated rats was less than that of the control group 1 week

TABLE I BODY WEIGHT, RIGHT VENTRICULAR SYSTOLIC PRESSURE (RVSP), VENTRICULAR HEART WEIGHT

After treatment	Body Weight (g)		RVSP (mmHg)		RV/(LV+IVS)	
	Control	MCT	Control	MCT	Control	MCT
1W	211.7±2.6 (n=13)	160.0±19.3* (n=12)	26.9±1.6 (n=13)	29.4±1.5 (n=14)	0.34±0.02 (n=13)	0.32±0.01 (n=14)
2W	259.2±5.8 (n=11)	229.5±5.1* (n=11)	28.3±2.4 (n=11)	40.7±0.8* (n=11)	0.35±0.03 (n=9)	0.45±0.03* (n=10)
3W	310.0±4.8 (n=14)	253.3±7.6* (n=13)	27.8±2.3 (n=14)	53.8±1.0* (n=13)	0.28±0.01 (n=14)	0.56±0.03* (n=12)

Values are mean±SEM. MCT=monocrotaline-treated rats (40 mg/kg). The data are assessed by unpaired *t*-test. **p*<0.05 vs control. RV: right ventricle, LV: left ventricle, IVS: interventricular septum.

Fig 1. A (Left): Cumulative concentration-response curves to KCl in the pulmonary arteries with endothelium. (○): control state, (●): 1 week after MCT treatment, (△): 2 weeks after treatment, (▲): 3 weeks after treatment. (n=8) The values are mean±SEM, **p*<0.05 vs control state, #*p*<0.01 vs control state. B (Right): Cumulative concentration-response curves to KCl in the pulmonary arteries without endothelium. (n=8) Abridgment is the same as in Fig 1A.

after treatment. Right ventricular systolic pressure (RVSP) in MCT-treated rats increased 2 weeks after treatment. Right ventricular hypertrophy occurred 2 weeks after treatment (Table I).

The Cumulative Concentration-response Curves to KCl and 5-HT

The sensitivity of the contractile response to KCl in the arteries with endothelium at 1 week after treatment was similar to that of the control group, but markedly increased at 2 and 3 weeks after treatment (Fig 1A). In the arteries without endothelium, the

sensitivity of the contractile response to KCl at 1 week was similar to that of the control, but slightly increased at 2 and 3 weeks (Fig 1B). When endothelium was removed, the cumulative concentration-response curves to KCl were already shifted to the left in the control state and at 1 week, and the concentration-response curves seemed to be slightly shifted to the left at 2 and 3 weeks after treatment. The sensitivity of the contractile response to 5-HT increased from 1 to 2 weeks after treatment; however, it decreased after 3 weeks in the arteries with endothelium (Fig 2A). The sensitivity of the

Fig 2. A (Left): Cumulative concentration-response curves to 5-HT in the pulmonary arteries with endothelium. (n=8) Abridgment is the same as in Fig 1A.
B (Right): Cumulative concentration-response curves to 5-HT in the pulmonary arteries without endothelium. (n=8) Abridgment is the same as in Fig 1A.

Fig 3. Cumulative concentration-response curves to KCl in the pulmonary arteries (○): with endothelium, (●): without endothelium. (n=8) The values are mean $\pm$ SEM, * $p < 0.05$ vs arteries with endothelium. # $p < 0.01$ vs arteries with endothelium.

Fig 4. Cumulative concentration-response curves to 5-HT in the pulmonary arteries (○): with endothelium, (●): without endothelium. (n=8) Abridgment is the same as in Fig 3.

contractile response to 5-HT at 1 week after treatment markedly increased compared to the control group in the arteries without

endothelium. However, this hypersensitivity was gradually reduced at 2 weeks post-treatment. The concentration-response curve at 3

Fig 5. A (Left): The change of right ventricular systolic pressure (RVSP) and EC₅₀ for KCl. (○): pulmonary artery with endothelium; (●): pulmonary artery without endothelium; (△)=RVSP. The values are mean ± SEM, *p < 0.05 vs pulmonary artery with endothelium. #p < 0.05 vs control state. B (Right): The change of right ventricular systolic pressure and EC₅₀ for 5-HT. Abridgment is the same as in Fig 5A.

Fig 6. A (Left): Cumulative concentration-response curves to acetylcholine (ACh) in pulmonary arteries. (○): control state, (●): 1 week after MCT treatment, (△): 2 weeks after treatment. (n=6) The values are mean ± SEM, *p < 0.05 vs control state, §p < 0.05 vs 1W. B (Right): Cumulative concentration-response curves to nitroglycerin (NTG) in pulmonary arteries. (n=6) Abridgment is the same as in Fig 6A.

weeks was similar to that of the control (Fig 2B).

The cumulative concentration-response curves to KCl were summarized for each week after MCT treatment (Fig 3). The sensitivity of the contractile response to KCl was increased by the removal of endothelium at 1 week after treatment, but, the procedure had no such effect at 2 or 3 weeks after treatment. Similarly, the cumulative concentration-response curves to 5-HT were summarized each week (Fig 4). The sensitivity of the contractile response to 5-HT was

increased by the removal of endothelium at 1 week after treatment, and to a lesser degree at 2 and 3 weeks after treatment as well.

The relation between the RVSP and EC₅₀ values for KCl and 5-HT for each week is shown in Figs 5A and 5B, respectively. These EC₅₀ values were similar at 2 and 3 weeks after MCT-treatment, in parallel with the development of pulmonary hypertension.

The Cumulative Concentration-Response Curves to ACh and NTG

The relaxation response to ACh at 1

Fig 7. The content of tissue cGMP in the pulmonary arteries stimulated by ACh or contracted by phenylephrine ($n=6$). The values are mean $\pm$ SEM * $p<0.05$ vs control state, § $p<0.05$ vs 1W.

week after treatment was similar to that of the control. The relaxation was markedly reduced at 2 and 3 weeks. The contractile response to a high concentration of ACh (10^{-5} mol/L) occurred at 3 weeks post-treatment (Fig 6A). The relaxation response to NTG at 1 week was similar to that of the control, but significantly reduced at 2 and 3 weeks after treatment (Fig 6B).

The Content of Tissue cGMP

The content of tissue cGMP in arteries stimulated by ACh or contracted by Phe was not reduced at 1 week after treatment. However, it was significantly reduced at 2 and 3 weeks (Fig 7).

DISCUSSION

The endothelium plays an important role in regulating the vascular tone by releasing EDRFs and endothelium-derived contracting factors (EDCFs)^{18,19} The endothelium attenuates the contractile response to vasoconstrictive agents with EDRF, released by high shear stress²⁰ or by agonists through their specific receptors. In the present study, the sensitivity of contractile responses to KCl and 5-HT increased with the removal of endothelium from the pulmonary arteries of control rats. Interestingly, this effect was reduced in the advanced stages of hypertension in the pulmonary arteries of MCT-treated rats (Figs 3 and 4). This is the first time vasoreactivity modulated by endothelium has been observed. Similarly, the relaxation

response to ACh was also reduced in the advanced stages of hypertension. The content of tissue cGMP was reduced when the pulmonary artery was stimulated by ACh or contracted by phenylephrine. These results suggest that the endothelial function, which is to release EDRF/NO, is impaired in the advanced stages of MCT-induced pulmonary hypertension. Previously, the pathological change in the endothelium was observed in the early stage of this model⁵ However, in the present study the relaxation response to ACh was not diminished in the early stages, and it decreased in the advanced stages of pulmonary hypertension. This finding is consistent with those of Altieri et al¹² The reason for this change in endothelial function is unknown. Similarly, such diminished relaxation occurred in systemic arteries isolated from spontaneously hypertensive rats (SHR)^{21,22} The impairment of endothelium-dependent relaxation is thought to be a secondary change due to hypertension²² and it may also be attributable to pulmonary hypertension in MCT-treated rats.

In addition, the relaxation response to NTG, which is metabolized to NO²³ was also reduced in the advanced stages of MCT-induced pulmonary hypertension (Fig 6B). This is consistent with the results of Wanstall et al¹³ They suggested that the activity of guanylate cyclase could be desensitized by increased EDRF in the pulmonary artery of MCT-treated rats. However, we disagree with this conclusion, because we suspect that in our study, the release of EDRF was reduced in the advanced stages. Further studies are needed to clarify the mechanism of the diminished relaxation of smooth muscle.

In the present study, the sensitivity of the contractile response to 5-HT was greater in the pulmonary arteries isolated from MCT-treated rats (Figs 2A and 2B). However, this sensitivity was less in the advanced stages than the early stages. This result differs from that of Wanstall et al, who observed an increase in the sensitivity of the contractile response to 5-HT between 2 and 4 weeks after treatment¹³ Several factors are thought to account for this discrepancy. The most apparent is that pulmonary arterial pressure was different in each study. Pulmonary hypertension occurred at 2 weeks

after treatment in our study, whereas it did not occur at the same stage in Wanstall's model. A similar reduction in the sensitivity to angiotensin II was reported by Altieri et al.¹² who suggested that diminished contractility was associated with a receptor defects, abnormalities in excitation-contraction coupling, or alterations in contractile proteins in vascular smooth muscle. Furthermore, the uptake of 5-HT into the endothelium was reduced, and the concentration of 5-HT was elevated in the pulmonary arteries of MCT-treated rats.²⁴ Why the sensitivity to 5-HT was reduced in the advanced stages in our study is still unclear; however, it may be due to down-regulation of 5-HT receptor in the smooth muscle.

The pathological role of endogenous endothelin-1, one of the EDCFs, was recently studied in MCT-treated rats.¹⁴ Endothelin-1 also contributes to the development of MCT-induced pulmonary hypertension. Several other factors, such as the products of the inflammatory cells or endocrine, may affect the progress of pulmonary hypertension in MCT-treated rats.

The sensitivity to the vasoconstrictors did not always increase in parallel with the development of pulmonary hypertension. Conversely, the endothelial function, which modulates vasoreactivity, and the relaxation response to EDRF/NO in smooth muscle were reduced with developed pulmonary hypertension at 2 and 3 weeks after treatment. In conclusion, the impairment of both the endothelial function and the relaxation response to EDRF/NO in smooth muscle may contribute to the hyperreactivity to vasoconstrictors in MCT-treated arteries, and may in part contribute to the development of MCT-induced pulmonary hypertension. However, it is not known whether impairment of the endothelium contributed to pulmonary hypertension in the present study. In addition, we cannot directly apply observations obtained in the large pulmonary arteries to the small resistant vessels in the pulmonary vascular tree. Further investigations are needed to clarify the mechanisms of MCT-induced pulmonary hypertension.

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