Role of inhibition of nitric oxide production in monocrotaline-induced pulmonary hypertension

RAJAMMA MATHEW, ELIZABETH S. GLOSTER, T. SUNDARARAJAN, CARL I. THOMPSON, GUILLERMO A. ZEBALLOS, AND MICHAEL H. GEWITZ

Departments of Pediatrics and Physiology, New York Medical College, Valhalla 10595; and Department of Pathology, State University of New York Health Science Center, Brooklyn, New York 11203

Mathew, Rajamma, Elizabeth S. Gloster, T. Sundararajan, Carl I. Thompson, Guillermo A. Zeballos, and Michael H. Gewitz. Role of inhibition of nitric oxide production in monocrotaline-induced pulmonary hypertension. J. Appl. Physiol. 82(5): 1493–1498, 1997.—Monocrotaline (MCT)-induced pulmonary hypertension (PH) is associated with impaired endothelium-dependent nitric oxide (NO)-mediated relaxation. To examine the role of NO in PH, Sprague-Dawley rats were given a single subcutaneous injection of normal saline [control (C)], 80 mg/kg MCT, or the same dose of MCT and a continuous subcutaneous infusion of 2 mg·kg−1·day−1 of molsidomine, a NO prodrug (MCT+MD). Two weeks later, plasma NO3− levels, pulmonary arterial pressure (Ppa), ratio of right-to-left ventricular weights (RV/LV) to assess right ventricular hypertrophy, and pulmonary histology were evaluated. The plasma NO3− level in the MCT group was reduced to 9.2 ± 1.5 μM (n = 12) vs. C level of 17.7 ± 1.8 μM (n = 8; P < 0.02). In the MCT+MD group, plasma NO3− level was 12.3 ± 2.0 μM (n = 8). Ppa and RV/LV in the MCT group were increased compared with C [Ppa, 34 ± 3.4 mmHg (n = 6) vs. 19 ± 0.8 mmHg (n = 8) and 0.41 ± 0.03 (n = 9) vs. 0.25 ± 0.008 (n = 8), respectively; P < 0.001]. In the MCT+MD group, Ppa and RV/LV were not different when compared with C [19 ± 0.5 mmHg (n = 5) and 0.27 ± 0.01 (n = 9), respectively; P < 0.001 vs. MCT]. Medial wall thickness of lung vessels in the MCT group was increased compared with C [31 ± 1.5% (n = 9) vs. 13 ± 0.66% (n = 9); P < 0.001], and MD partially prevented MCT-induced pulmonary vascular remodeling [22 ± 1.2% (n = 11); P < 0.001 vs. MCT and C]. These results indicate that a defect in the availability of bioactive NO may play an important role in the pathogenesis of MCT-induced PH.

molsidomine; pulmonary vascular remodeling

ENDOTHELIAL DYSFUNCTION is thought to underlie various clinical and experimental forms of pulmonary hypertension. The absence of a relaxation response to nitroglycerin in pulmonary microvessels in vitro is reported to be associated with a high mortality rate in children with congenital heart defect and pulmonary hypertension. Reduced nitric oxide (NO) generation in the lungs has been shown in this clinical group (8, 23). Impaired endothelium-dependent NO-mediated relaxation has been shown in isolated pulmonary arteries (PA) from humans suffering from Eisenmenger syndrome (9). However, there are conflicting reports about NO production in experimental models of pulmonary hypertension. There have been findings suggestive of increased NO production in chronic hypoxia-induced pulmonary hypertension in rats (16, 36) and monocrotaline (MCT)-induced pulmonary hypertension (20), whereas other studies show impaired NO-mediated relaxation in PA from rats subjected to chronic hypoxia (1). Our previous studies indicate a decrease in NO production in MCT-induced pulmonary hypertension (24). The reasons for these differences are not yet clear.

NO produced by the vascular endothelial cells plays an important role in the modulation of vascular tone and structure. In addition to its vasodilator role, NO inhibits the production of various growth factors, including endothelin-1 (ET-1), and inhibits smooth muscle proliferation by a guanosine 3′,5′-cyclic monophosphate-dependent mechanism (5, 12, 26). Short-term NO inhalation reduces pulmonary arterial pressure (Ppa) in various forms of pulmonary hypertension (18, 30, 31), whereas prolonged inhalation of NO attenuates pulmonary vascular remodeling induced by chronic hypoxia in adult and newborn rats (19, 32). Based on these observations coupled with our previous data, we postulated that in the absence of an inhibitory influence of NO, endothelium-derived contracting factors and other growth factors may have the potential to exert vasoconstrictive and mitogenic effects on pulmonary vessels, thus contributing to the pathogenesis of MCT-induced pulmonary hypertension.

To test this hypothesis, we administered a continuous subcutaneous infusion of molsidomine (MD), a NO donor, to rats injected with MCT. Ppa, right ventricular hypertrophy, and pulmonary vessels were evaluated 2 wk after MCT/vehicle injection. Plasma nitrate (NO3−) levels were measured as an index of NO generation in the different experimental groups.

MATERIALS AND METHODS

Experimental Groups

Male Sprague-Dawley rats (180–200 g, Charles River Laboratories, Wilmington, MA) were divided into six groups. Group I (control [C]) received a single subcutaneous injection of normal saline. Group Ia (C+MD) received normal saline subcutaneously and a continuous infusion of MD (2 mg·kg−1·day−1 for 14 days) via an osmotic minipump. Group II (MCT) received MCT (80 mg/kg sc). Group IIa (MCT+V) received MCT (80 mg/kg sc) and a continuous infusion of an equivalent amount of the vehicle used for dissolving MD (20% ethanol) for 14 days. Group III (MCT+MD) received MCT (80 mg/kg sc) and a continuous infusion of MD (2 mg·kg−1·day−1 for 14 days). MCT is metabolized in the liver to dehydromonocrotaline, an active form that induces lung injury within 1–3 days of administration and causes a delayed onset of pulmonary hypertension (25, 34). Therefore, to ascertain whether the effects of MD were secondary to an interference with MCT metabolism in liver, a group of rats was started on MD infusion 48 h after MCT injection and continued for 12 days.
Measurements of Plasma NO$_3$ Levels

Heparinized blood was collected from the rats on the day of the hemodynamic study. Plasma samples were prepared and frozen (−20°C) until assayed. Plasma NO$_3$ levels were measured by the chemiluminescence method (6, 7). Briefly, after reduction of plasma NO$_3$ in vanadium chloride at 90°C in a tube capped with a rubber seal, the NO released to the headspace was injected into a NO analyzer (model 270B; Sievers Instruments, Boulder, CO). The analog signals from the reaction of NO with ozone were recorded and analyzed by a computerized integrator (model HP396; Hewlett-Packard). Determination of NO$_3$ content in the sample was made by regression analysis using the slope of the standard curve. The standard curve for NO$_3$ was linear over the range of 0–80 µM. All sample concentrations of NO$_3$ fell within this range.

Evaluation of Pathophysiology

Hemodynamic Data. As previously described (22), rats were anesthetized with 60 mg/kg pentobarbital sodium. The trachea was exposed through a skin incision in the neck, cannulated with PE-240 tubing, and ventilated at 60–70 breaths/min in room air with a tidal volume of 0.83 ml/100 g body wt. The chest was opened, and PE-50 tubing was inserted into the right ventricle (RV) and advanced to the PA to record Ppa on a Grass polygraph (model 7E). When it was not possible to advance the catheter into the PA, RV pressure was recorded, and RV systolic pressure was used for statistical analysis. If signs of excessive bleeding were detected during the pressure measurement, that pressure record was not used for statistical analysis. At the end of the pressure recording, the left atrial appendage was cut for drainage, and PE-160 tubing was inserted into the PA via the RV. The lungs were perfused with phosphate buffer (pH 7.0) to remove blood. Heart and lungs were dissected free and preserved in 10% buffered formaldehyde for lung histology and the assessment of RV hypertrophy (RVH).

Assessment of RVH. The hearts were removed from formaldehyde after 1 wk. Ventricles were trimmed, and atria were discarded. The free wall of the RV was separated from the left ventricle and septum (LV) and then weighed. The ratio of RV to LV was calculated to assess RVH. RV and LV weights were also expressed in milligrams per gram of total body weight (TBW) to assess the effects of MCT and MD on both RV and LV.

Evaluation of lung vessels. Lungs were embedded in paraffin blocks, and 4-µm sections were made and then stained with Verhoeff's elastic stain and hematoxylin and eosin. The resistance PA were identified as vessels with two clearly defined elastic laminae, with a layer of smooth muscle cells between the two laminae. The percent wall thickness (%WT) of arteries (diameter, 15–150 µm) was calculated by using the following formula as described previously (21, 25): %WT = 2 × WT/ED × 100. The thickness of medial wall (WT) was measured under the microscope as the distance between the external and internal elastic laminae, as seen with the use of a calibrated eyepiece. ED was measured as the diameter of the external lamina. For each rat, ~15–20 vessels were counted, and an average was calculated. The vessels that were not close to round or oval in shape were not measured. Medial WT and the ED measurements were made at several points for each vessel, and an average was calculated.

Drugs

MCT and MD were obtained from Trans World Chemical, Rockville, MD, and Biomol Research Laboratory, Plymouth Meeting, PA, respectively. Vanadium chloride was purchased from Aldrich, Milwaukee, WI.

Statistical Analysis

The results are expressed as means ± SE. Student's t-test and one- and two-way analyses of variance for multiple responses were performed, using Scheffe's test, as appropriate. A P value of <0.05 was considered significant.

RESULTS

Weight Gain

As shown in Fig. 1, rats in group II (MCT) lost weight 7 days after the MCT injection. Thereafter, weight gain in this group was not significantly different compared with the control group. Weight gain in the MCT + MD group (group III) was significantly lower at each time point when compared with control. Compared with the MCT group, the weight gain in the MCT + MD group was not significantly different.
Systolic Pressure in Systemic Artery

As shown in Table 1, treatment with MCT or MD did not affect systolic pressure in systemic artery in any of the groups.

Plasma NO$_3^-$ Levels

The plasma NO$_3^-$ level in the MCT group (group II) was significantly reduced compared with the control group (Fig. 2). In the MCT + MD group (group III) the plasma NO$_3^-$ level was higher compared with the MCT group, but it did not reach statistical significance. In the group of rats treated with MD only (group Ia, n = 4), plasma NO$_3^-$ level (13.3 ± 1.6 µM) was not significantly lower compared with controls.

Ppa

The MCT-treated group showed significant pulmonary hypertension compared with controls. MD significantly attenuated MCT-induced pulmonary hypertension (Fig. 3). MD infusion, started 48 h after MCT injection (group IIIa), was equally effective in attenuating MCT-induced pulmonary hypertension (Ppa, 18 ± 2 mmHg; n = 3). MD alone (group Ia) had no effect on Ppa (17 ± 0.6 mmHg, n = 4). Similarly, the administration of the vehicle in the MCT group (group IIa) did not alter MCT-induced pulmonary hypertension (Ppa, 35 ± 5 mmHg; n = 4).

RVH

Figure 4 shows a significant increase in RV/LV ratio as an index of RVH in the MCT group (group II) compared with controls (group I) and a significant reduction of RVH in the MCT + MD group (group III). MD infusion started 48 h after MCT injection (group IIIa) also showed a significant attenuation of RVH (RV/LV 0.23 ± 0.009, n = 5, P < 0.001 vs. MCT group). MD treatment in the control group (group Ia) did not affect the RV/LV ratio (0.25 ± 0.009, n = 5). The administration of vehicle did not prevent MCT-induced RVH (RV/LV 0.37 ± 0.01, n = 4) in group IIa.

The RV weight expressed as RV/TBW (in mg/g) confirmed significant RVH after MCT administration.
and a significant reduction of RVH after MD treatment (Table 1). The LV weight (LV/TBW) was not influenced by MCT, MD, or the vehicle (Table 1).

Histology of Lung Vessels

Pulmonary arterioles in lungs from MCT-treated rats (group II) showed a significant increase in medial WT compared with the control group (group I). In the MCT + MD-treated group (group III), there was a significant attenuation of medial WT compared with the MCT group. However, compared with the control group, the MCT + MD group exhibited increased medial WT (Fig. 5).

DISCUSSION

A single subcutaneous injection of MCT in rats induces progressive pulmonary endothelial injury, resulting in pulmonary hypertension and cardiopulmonary changes by 10–14 days. In a previous study, we showed that pulmonary hypertension and RVH are not evident at 1 wk post-MCT (24), and in this group, plasma NO\textsubscript{3} levels (20 ± 3.4 µM, n = 5) were within the normal range. The present study demonstrates that 2 wk post-MCT, there is reduction in plasma NO\textsubscript{3} levels concomitant with pulmonary hypertension and RVH. The administration of MD, a NO prodrug, prevents MCT-induced pulmonary hypertension and RVH. The associated pulmonary vascular remodeling is attenuated, but not completely inhibited, by the dose of MD that we used. It is possible that a higher dose of MD may have had a more pronounced effect on vascular remodeling.

MD, a sydnonimine compound, first undergoes an enzymatic conversion to 3-morpholinosydnonimine (SIN-1), in the liver. SIN-1, in turn, is catalyzed by hydroxyl ions in a pH-dependent manner to an active compound SIN-1A, which, in the presence of oxygen, liberates NO and superoxide anions nonenzymatically. It has been shown in vitro that a small quantity of hydroxyl ions is formed in the presence of NO and superoxide anions. In addition to scavenging NO, superoxide anions enhance the rate of decomposition of SIN-1A, thus influencing the rate of NO formation (4, 10, 15, 27, 29). Therefore, the effect of MD in vivo is dependent on the relative quantities of NO and superoxide anions produced. It is not clear how NO, once released from its parent compound, is transported to the pulmonary vessels without being degraded by superoxide anions and hemoglobin. One could speculate that S-nitrosothiols or a similar compound may play a role (17).

The dose of MD used in this study did not affect systemic pressure in any of the groups and also had no effect on the pulmonary vasculature of the control rats. The vehicle (20% ethanol) used for dissolving MD did not alter MCT-induced cardiopulmonary changes. Because both MCT and MD are metabolized in the liver and the lung injury occurs within 1–3 days of MCT administration, one could argue that MD may have interfered with MCT metabolism, thus resulting in attenuation of MCT-induced pulmonary hypertension. The fact that the MD infusion started 48 h after MCT injection also resulted in a similar inhibition of MCT-induced pulmonary hypertension indicates that the attenuation of pulmonary hypertensive changes was secondary to its effects on hypertensive vessels and not a result of any interference with MCT metabolism in the liver.

Prolonged NO inhalation has been shown to attenuate chronic hypoxia-induced pulmonary vascular remodeling and pulmonary hypertension in rats (19, 32). Because chronic hypoxia-induced pulmonary hypertension is associated with vasoconstriction, polycythemia and subsequent vascular remodeling, NO inhalation may have prevented, in large part, the vasoconstrictor component of chronic hypoxia, thereby influencing vascular remodeling. Polycythemia, however, is not present in MCT-induced pulmonary hypertension (25). Although enhanced contractility in PA has been shown 4 days post-MCT, the response returns to normal by 7 days post-MCT (2), before the onset of pulmonary hypertension. Similar to our previous findings, Altiere et al. (2) also have shown diminished relaxation responses 2 wk post-MCT, when pulmonary hypertension is already established. Thus the inappropriate vasoconstriction observed early in the course is unlikely to be a contributory factor in the development of MCT-induced pulmonary hypertension.

Injury to pulmonary vascular endothelium plays a key role in the pathogenesis of MCT-induced pulmonary hypertension. The endothelial injury is followed by hyperplasia and hypertrophy of smooth muscle cells in muscular arteries, neomuscularization, and extracellular matrix deposits (35). Much evidence is accumulating to suggest that multiple growth factors contribute to the regulation of cellular responses in MCT-induced pulmonary hypertension (3). Tissue and plasma levels of ET-1, a contractile and mitogenic factor, are also elevated in various forms of pulmonary hypertension, including the MCT model (13, 24, 33). It is well established that NO counters vasoconstriction and mitogenesis via a guanosine 3',5'-cyclic monophosphate-
dependent pathway. Recent studies indicate that NO, via the same signal transduction mechanism, mediates the induction of apoptosis in vascular smooth muscle cells (28). In this context, it is interesting to note that vasoconstriction does not play a role in the pathogenesis of MCT-induced pulmonary hypertension. Therefore, the attenuating effects of MD in MCT-induced pulmonary hypertension and vascular remodeling may be related to antiproliferative, antihypertrophic, and perhaps apoptotic properties of NO. It is also noteworthy that the 10–25% reduction of platelets in MCT-injected rats results in attenuation of pulmonary hypertension and RVH, indicating a role for platelets in this model (11). Platelets, by releasing platelet-derived growth factor, are known to play a critical role in cell migration (14). Thus NO could also mediate its effects by inhibiting platelet aggregation, thus inhibiting cell migration and vascular remodeling in this model of pulmonary hypertension.

Although MCT-induced pulmonary hypertension may not be an ideal model for pulmonary hypertension, the endothelial dysfunction seen with MCT appears to be similar to that seen in most forms of pulmonary hypertension. Our data support a role for the NO signaling pathway in the pathogenesis of MCT-induced pulmonary hypertension. The common denominator in most forms of pulmonary hypertension may be a defect in the availability of bioactive NO in the presence of hemodynamic stress and external stimuli. However, it is not yet known whether the defect in NO availability lies in its synthesis, release, or rapid degradation.

In summary, MCT administration in rats results in low plasma NOX levels and pulmonary hypertension. A continuous infusion of MD, a NO produg, attenuates MCT-induced pulmonary hypertension and associated cardiopulmonary changes. These results imply a significant role for NO in the pathogenesis of MCT-induced pulmonary hypertension.

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congenital heart defects and pulmonary hypertension (Abstract).


