Endothelin-1 is elevated in monocrotaline pulmonary hypertension

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Frasch, H. Frederick, Carol Marshall, and Bryan E. Marshall. Endothelin-1 is elevated in monocrotaline pulmonary hypertension. Am. J. Physiol. 276 (Lung Cell. Mol. Physiol. 20): L304–L310, 1999.—These studies document striking pulmonary vasoconstrictor response to nitric oxide synthase (NOS) inhibition in monocrotaline (MCT) pulmonary hypertension in rats. This constriction is caused by elevated endothelin (ET)-1 production acting on ETA receptors. Isolated, red blood cell plus buffer-perfused lungs from rats were studied 3 wk after MCT (60 mg/kg) or saline injection. MCT-injected rats developed pulmonary hypertension, right ventricular hypertrophy, and heightened pulmonary vasoconstriction to ANG II and the NOS inhibitor N\textsuperscript{G}-monomethyl-L-arginine (L-NMMA). In MCT-injected lungs, the magnitude of the pulmonary pressor response to NOS inhibition correlated strongly with the extent of pulmonary hypertension. Pretreatment of isolated MCT-injected lungs with combined ETA (BQ-123) plus ETB (BQ-788) antagonists or ETA antagonist alone prevented the L-NMMA-induced constriction. Addition of ETB antagonist reversed established L-NMMA-induced constriction; ETB antagonist did not. ET-1 concentrations were elevated in MCT-injected lung perfusate compared with sham-injected lung perfusate, but ET-1 levels did not differ before and after NOS inhibition. NOS inhibition enhanced hypoxic pulmonary vasoconstriction in both sham- and MCT-injected lungs, but the enhancement was greater in MCT-injected lungs. Results suggest that in MCT pulmonary hypertension, elevated endogenous ET-1 production acting through ETA receptors causes pulmonary vasoconstriction that is normally masked by endogenous NO production.

METHODS

Animals. Virus-free male Sprague-Dawley rats obtained from Charles River were used for these experiments, and the protocols were approved by our institutional review board. MCT (Sigma) was dissolved in 1 N HCl. The pH was neutralized with 0.5 N NaOH, and the volume of the solution was adjusted with phosphate-buffered saline (PBS; pH 7.4) to achieve a concentration of 30 mg/ml. Animals (225–250 g) were given a single subcutaneous injection of MCT (60 mg/kg) or were given an equivolume injection of PBS (2 ml/kg). Studies were performed 20–21 days after MCT or sham injection.

Isolated, perfused lung. Rats were anesthetized with an intraperitoneal injection of 60 mg/kg of pentobarbital sodium. A ventral midline neck incision was made, and the trachea was isolated and intubated with a blunt 17-gauge stainless steel needle. The rats were ventilated with room air with a Harvard volume-controlled ventilator (10 ml/kg, 60 strokes/min).

The chest was opened via a midline sternotomy, and the heart and lungs were exposed. Heparin (200 U) was injected into the left ventricle. A suture (3-0 silk) was looped around the main pulmonary artery and ascending aorta and another was put around the ventricles. The animal was exsanguinated by left ventricle puncture, and blood was collected into a 15-ml modified polystyrene centrifuge tube (Corning). The inspiratory gas was changed to a mixture of 21% O\textsubscript{2}-5% CO\textsubscript{2}-balance N\textsubscript{2} (normoxic gas mixture). A large-bore cannula was inserted through the apex of the left ventricle, advanced into the left atrium, and secured by suture. Another cannula was secured in the main pulmonary artery through an incision in the right ventricle. Perfusion of the lungs was initiated by pumping a steady flow of perfusate into the pulmonary arterial cannula, gradually increasing the rate to 0.06 ml·min\textsuperscript{-1}·g\textsuperscript{-1}. The lungs were washed free of blood with ~50 ml of perfusate. Perfusate was then recirculated (total...
volume 50 ml) for the duration of the experiment. The heart and lungs remained in situ for the experiment. The blood that was collected was spun at 2,500 rpm for 20 min, and the packed red blood cell fraction (3.5–4 ml) was slowly added to perfusate. A Masterflex pump (Cole Parmer) with a modified flow controller enabled a precisely calibrated regulation of flow. A 20-ml air reservoir or windkessel distal to the pump dampened pulsations to <0.1 cmH2O. Transducers placed near the cannulas allowed measurement of pulmonary arterial and left atrial pressures; left atrial pressure was kept at 0 cmH2O by adjusting the height of the reservoir. Pressures were recorded continuously on a Gould chart recorder. The perfusion apparatus was maintained at 37–39°C with a heat lamp.

Physiological salt solution contained the following (in mM): 131 NaCl, 4.7 KCl, 1.17 MgSO4, 22.61 NaHCO3, 1.18 K2HPO4, 3.2 CaCl2, and 10.0 glucose. In addition, 1.6 mU/ml of insulin, 5 × 10−6 g/ml of medofenamate, and 4% (wt/vol) BSA were added to the perfusate. With the addition of 3.4–5 ml of packed red blood cells, hematocrit in the perfusate was 7–8%.

When pulmonary arterial pressure (PAP) stabilized (5–10 min after addition of red blood cells), a bolus (0.3 µg) of ANG II was injected upstream of the lung as a test for pulmonary vascular reactivity and to “prime” the vasomotor tone of the isolated lungs. Prior to the peak PAP response was taken as the peak PAP recorded minus the pre-ANG II baseline PAP.

The effect of NOS inhibition.

In 18 MCT-injected and 8 control (sham-injected) rats, the effect of NOS inhibition on baseline PAP was evaluated. After equilibration, the NOS inhibitor N3-monomethyl-L-arginine (L-NMMA; Cyclophsp Biochem, Salt Lake City, UT) was added to the perfusate to a final concentration of 3 × 10−6 M. Effects were recorded for 30 min.

The effect of ETA or ETB antagonist after NOS inhibition. In 10 of the 18 MCT-treated rats used to study the effects of NOS inhibition, the ability of either ETA or ETB blockade to reverse the L-NMMA-induced constriction was studied. After a 30-min perfusion with L-NMMA, the ETA-receptor antagonist BQ-123 (5 × 10−6 M) was added in five lungs. In five other lungs, the ETB antagonist BQ-788 (5 × 10−6 M; both from RBI, Natick, MA) was added. PAP after ETA or ETB blockade was monitored for 30 min. Afterward, sodium nitroprusside (10−5 M) was added as a bolus to the perfusate to relieve active constriction, and the resulting PAP was measured.

The effect of pretreatment with combined ETA plus ETB antagonists or ETA alone. In four MCT-treated rats, the ability of combined ETA plus ETB blockade to reverse L-NMMA-induced pulmonary vasoconstriction was examined. The ETA-selective antagonist BQ-123 (5 × 10−6 M) and ETB-selective antagonist BQ-788 (5 × 10−6 M) were added to perfusate 15 min before addition of L-NMMA.

The effect of pretreatment with an ETA antagonist alone was also evaluated. In isolated lungs from four MCT-treated rats, BQ-123 (5 × 10−6 M) was added 15 min before L-NMMA.

Pulmonary ET-1 production. In nine MCT-treated rats and seven control rats, the ANG II response was taken as the peak PAP recorded minus the pre-ANG II baseline PAP. The ET-1 response was taken as the peak PAP recorded minus the pre-ANG II baseline PAP.

For determination of right ventricular hypertrophy. After the experiments, the hearts were excised. The atria were removed, and the right ventricular free wall was dissected from the left ventricle and septum; both were blotted and weighed for determination of the ratio of weights of right ventricle to left ventricle plus septum (RV/(LV+S)), an index of right ventricular hypertrophy.

ET-1 assay. ET-1 levels in perfusate were measured with a commercial radioimmunoassay (Peninsula Laboratories, Belmont, CA) following the company’s protocol. Briefly, previously frozen perfusate samples were acidified with equal volumes of 1% trifluoroacetic acid (buffer A), and the peptide was extracted with 200 mg of C18 solid-phase extraction cartridges activated with 1 ml of 60% acetonitrile in buffer A (buffer B) and then washed three times with 3 ml of buffer A. Samples were loaded onto the columns and washed two times with 3 ml of buffer A. The peptide was eluted with 3 ml of buffer B into polypropylene tubes. Eluant was evaporated with a centrifugal vacuum concentrator (Savant). The residue was dissolved in buffer and assayed in duplicate for ET-1 according to the manufacturer’s instructions. The detection limit was 0.5 pg/ml of sample, and recovery of ET-1 spiked in perfusate averaged 65%.

Statistical analysis. Statistical analyses were performed with the use of SigmaStat for Windows (Jandel Scientific). Comparisons between control and MCT-treated groups were made with an unpaired t-test, and comparisons before and after a treatment were made with a paired t-test. Variables in the MCT-injected groups were often not normally distributed. In these cases, Wilcoxon’s signed rank or Mann-Whitney rank sum tests of differences in median values were performed. Strength of association between two variables was evaluated with Pearson’s correlation. A P value < 0.05 was considered significant. Data are presented as means ± SE.

RESULTS

Pulmonary hypertension and right ventricular hypertrophy. Injection of rats with MCT led to pulmonary hypertension and right ventricular hypertrophy. The RV/(LV+S) (Fig. 1A) for all animals used in these studies was 0.288 ± 0.006 for sham-injected rats (n = 14) and 0.487 ± 0.015 for MCT-injected rats (n = 36; P < 0.0001). Isolated lung vascular resistance normalized by body weight (Fig. 1B) was elevated in MCT-injected rat lungs (506 ± 43 cmH2O·min·g·ml−1;
n = 36) compared with sham-injected lungs (307 ± 6 cmH₂O·min·g·ml⁻¹; P < 0.005; n = 14).

Response to ANG II. Isolated lungs from MCT-injected rats exhibited heightened responsiveness to ANG II (Fig. 2A). The change in PAP over baseline in response to a bolus injection of 0.3 µg of ANG II was 20.2 ± 2.6 cmH₂O in MCT-injected lungs (n = 36) and 3.9 ± 0.4 cmH₂O in sham-injected lungs (P < 0.0001; n = 14).

Response to L-NMMA. Addition of the NOS inhibitor L-NMMA to perfusate increased baseline PAP in MCT-injected lungs (Fig. 2B); however, there was wide variability in the magnitude of the response. After 30 min of perfusion with 3 × 10⁻⁴ M L-NMMA, change in PAP over baseline was 12.8 ± 3.2 cmH₂O in MCT-injected lungs (n = 28) and 1.6 ± 0.4 cmH₂O in sham-injected lungs (P < 0.0001; n = 14).

The magnitude of pressor response to NOS inhibition correlated with the extent of pulmonary hypertension. Figure 3 displays the magnitude of the pressure response after 30 min of perfusion with L-NMMA (3 × 10⁻⁴ M) vs. initial baseline PAP in isolated, perfused Sham (n = 8) and MCT-injected (n = 18) lungs. A positive and significant correlation (Pearson's product moment) exists for MCT-injected but not for Sham lungs.
Effect of posttreatment with BQ-123 or BQ-788. NOS inhibition of MCT-injected rat lungs produced an initial rapid (3–5 min) increase in baseline PAP followed by a slower, steady increase. The increase was significantly reversed after the addition of an ETₐ antagonist, but the increase was unaltered by addition of an ETₐ antagonist. Figure 4 shows the time course of normalized PAP change for 30 min after administration of 3 × 10⁻⁴ M NOS inhibitor L-NMMA in MCT-injected rat lungs and the subsequent response to 5 × 10⁻⁶ M ETₐ antagonist BQ-123 (n = 5) or the ETₐ antagonist BQ-788 (n = 5). Baseline PAP (PAP before addition of L-NMMA) was 30.0 ± 3.1 cmH₂O in the BQ-123 group and 22.8 ± 1.5 cmH₂O in the BQ-788 group. Addition of BQ-123 significantly (P < 0.01) reversed the L-NMMA-induced rise in PAP. In contrast, addition of BQ-788 did not alter the L-NMMA-induced increase in PAP.

After addition of sodium nitroprusside to a final concentration of 10⁻⁵ M, PAP declined to below initial baseline levels in both MCT (P < 0.02) and sham-injected (P < 0.005) lungs.

Effect of pretreatment with combined BQ-123 plus BQ-788 or BQ-123 alone. Pretreatment of perfused MCT lungs with BQ-123 plus BQ-788 prevented the L-NMMA-induced increase in PAP (Fig. 5). Pretreatment with the ETₐ-specific antagonist BQ-123 alone was equally effective. Addition of BQ-123 or BQ-123 plus BQ-788 did not alter baseline PAP, which was 21 ± 3.1 cmH₂O in the former (BQ-123) and 29 ± 4.8 cmH₂O in the latter group (BQ-123 plus BQ-788; P = 0.18). Transient but insignificant (P = 0.19 and P = 0.17) increases in PAP of 3.5–4 cmH₂O occurred at 5 min after addition of L-NMMA. By 30 min, PAP returned to baseline in both groups.

Perfusate ET-1 concentrations. ET-1 concentrations were significantly higher in MCT-treated rat lung recirculating perfusate compared with control lung perfusate, but the concentrations did not differ before or after L-NMMA infusion in either group. Figure 6 shows that ET-1 levels in perfusate of sham-injected rats (n = 7) were 0.18 ± 0.02 and 0.26 ± 0.04 pg/ml before and 30 min after L-NMMA addition, respectively. In MCT-treated rat lungs (n = 9), ET-1 levels were 13.0 ± 5.8 and 9.5 ± 5.4 pg/ml, respectively. These levels were significantly greater (P < 0.05 and P < 0.005, respectively) than corresponding levels in sham-injected rat lung perfusate. ET-1 levels before and after L-NMMA infusion were not significantly different in MCT lungs. ET-1 levels did not correlate with the severity of pulmonary hypertension in MCT lungs (P = 0.15).

Hypoxic response. Inhibition of NOS enhanced the hypoxic response in both control and MCT-injected rat lungs, but the effect was greater in the MCT group (Fig. 7). In control rats (n = 6), hypoxic responses measured before and 45 min after L-NMMA addition were 8.9 ± 1.1 and 25.3 ± 1.7 cmH₂O, respectively (P = 0.0001). In MCT-injected rats (n = 9), the hypoxic responses were 13.8 ± 3.1 cmH₂O before and 50.4 ± 6.0 cmH₂O after L-NMMA (P = 0.001). Before L-NMMA, the hypoxic responses were not significantly different between the sham- and MCT-injected groups (P = 0.2). However, the differences were significant between the two groups after L-NMMA addition (P < 0.05). In these MCT-treated rat lungs, a strong correlation also existed between the L-NMMA response and the initial baseline PAP (r = 0.90; P < 0.005).

Pulmonary edema was not evident despite these elevated pressures. The hypoxic vasoconstrictor response was totally reversible in both sham and MCT groups on return to normoxic ventilation. There were no significant differences in PAP before and after hypoxia in sham-injected (P = 0.57) and MCT-injected (P = 0.12) rats, and no changes in airway pressure were observed. There was no detectable edema formation in the MCT lungs. The lung wet-to-dry weight ratio was 5.70 ± 0.52 in the MCT group compared with 4.84 ± 0.11 in the sham group (P = 0.16).

DISCUSSION

A reasonable explanation for the results presented here is that both endogenous endothelial NO and ET-1...
production are elevated in MCT pulmonary hypertension, and the extent of upregulation is proportional to the severity of pulmonary hypertension. Endogenous NO production masks a constriction that would ensue from elevated ET-1 production acting on ETA receptors. This constriction is unmasked by inhibition of NOS. It thus appears that two potent vasoactive compounds of opposite effects are upregulated and oppose each other for control of pulmonary vascular tone.

In the chronic hypoxia rat model of pulmonary hypertension, NOS inhibition also leads to significant elevation of pulmonary vascular resistance compared with that in control lungs (1, 6, 14, 15). Oka et al. (15) searched for a vasoconstrictor that was suppressed by endogenous NO production, but they could not attribute their results to ET-1 or any common constrictor. More recently, however, the same group (14) found combined ETA and ETB receptor-mediated ET-1 constriction unmasked by NOS inhibition. The authors hypothesized that their conflicting results are due to differences in blood versus buffer perfusion.

Our observations of hyperresponsiveness to NOS inhibition in MCT pulmonary hypertension originally led to the hypothesis that the mechanism was mediated through ETb receptors. ETb receptors localized on vascular endothelium and smooth muscle mediate both vasoconstriction and vasorelaxation, the latter apparently through a coupling with stimulated NO release (24). We reasoned that NOS inhibition eliminated this relaxation pathway and thus unmasked a vasoconstrictor response. However, addition of the selective ETb-receptor antagonist BQ-788 failed to affect the L-NMMA-induced vasoconstriction (Fig. 4). On the other hand, blockade of ETA receptors with BQ-123 significantly reversed the constriction caused by NOS inhibition. On the basis of these data, it appears that NOS inhibition in MCT pulmonary hypertension unmasks vasoconstriction by endothelin acting on the ETA receptor.

Data from perfused normal rat lungs suggest that both ETA and ETB blockade are required to inhibit ET-1-induced pulmonary vasoconstriction. Sato et al. (22) found that although BQ-788 alone had no inhibitory effect, combined BQ-788 plus BQ-123 was more
effective than BQ-123 alone in blunting ET-1-induced increases in pulmonary vascular resistance. Because ET<sub>B</sub> receptors play an important role in the pulmonary clearance of ET-1 (3), Sato et al. (22) proposed that elimination of this clearance function by BQ-788 could explain their results. If ET-1 vasoconstriction is mediated by both ET<sub>A</sub> and ET<sub>B</sub> receptors, then an apparent lack of effect of BQ-788 might be explained as a balance between suppression of ET<sub>B</sub>-mediated vasoconstriction and an enhanced ET<sub>A</sub> constriction, made possible by increased circulating levels of ET-1.

Our data in Fig. 4 demonstrate that vasoconstriction caused by NOS inhibition in MCT pulmonary hypertension is reversed by ET<sub>A</sub> blockade but is unaffected by ET<sub>B</sub> block. However, it remains possible that ET<sub>B</sub> receptors play a complementary role similar to that demonstrated by Sato et al. (22) in normal rat lungs. Therefore, we compared ET<sub>A</sub>-receptor antagonism alone with combined ET<sub>A</sub>- and ET<sub>B</sub>-receptor antagonism on the inhibition of L-NMMA-induced pulmonary vasoconstriction. As demonstrated in Fig. 5, pretreatment with combined BQ-123 plus BQ-788 completely prevented the increase in resistance caused by NOS inhibition. However, pretreatment with BQ-123 alone was equally effective in preventing the increase in pulmonary vascular resistance.

It thus appears that in the MCT model of pulmonary hypertension, vasoconstriction induced by NOS inhibition is mediated exclusively through the ET<sub>A</sub> receptors. This contrasts with normal rat lung and the chronic hypoxia model, in which constriction is mediated by mixed ET<sub>A</sub> and ET<sub>B</sub> receptors. It is possible that a change in the role of ET receptors occurs in rat lungs after MCT injection; however, any elaboration on this point would be purely speculative without further investigation.

Our data in Fig. 4 showing a reversal of the increase in PAP after addition of BQ-123 are consistent with the report by Warner et al. (28). These authors demonstrated a slow reversal in established (systemic) constrictor response to ET-1 infusion after addition of BQ-123. The time course in pressure increase after ET-1 infusion and subsequent pressure decrease after BQ-123 are both similar to the time courses shown in Fig. 4. Warner et al. suggested that a large molar excess of receptor antagonist prevents new binding of ET-1 after receptor externalization. Thus the observed slow reversal of established constriction is most likely explained as a function of the rate of receptor recycling. Newly externalized receptors are bound by the antagonist BQ-123 rather than by ET-1; therefore, vasoconstriction is diminished over time.

In these experiments, we found a significant correlation in MCT-treated lungs between the severity of pulmonary hypertension and responses to NOS inhibition (Fig. 3). This suggests enhanced vasoactivity in response to MCT as the severity of hypertension increases. It is not known if this represents a change in function or is a consequence of pulmonary vascular remodeling. For example, it is possible that neomuscularization of previously nonmuscular small pulmonary arteries is responsible for this observed enhanced agonist response.

It should also be pointed out that these studies do not address the important issue of cause and effect; that is, whether upregulation of ET is involved in the pathogenesis of MCT pulmonary hypertension or is secondary to it. ET is a vascular smooth muscle mitogen (6); however, these studies do not shed light on whether upregulation of ET-1 is implicated in vascular remodeling in this model of pulmonary hypertension.

Further insight might be gained by correlating the time course of ET upregulation after MCT injection with pulmonary vascular remodeling. We chose to study rats 3 wk after MCT injection, as have numerous other investigators. At this time point, pulmonary hypertension is well developed, but mortality is low. Pulmonary vascular reactivity varies with time after MCT injection (20, 25); therefore, it is reasonable to expect that NOS and ET-1 regulation differ at other time points.

These studies address a question regarding the acute interaction between NOS activity and ET-1 release. We investigated the possibility that inhibition of endothelial NO production was related to the stimulation of ET-1 release. The data in Fig. 6 demonstrate that this is not the case; the measured ET-1 levels in perfusate were elevated (compared with control lungs) before L-NMMA addition and did not increase in response to L-NMMA. Thus it appears in this model that ET-1 vasoconstriction is masked by endogenous NO release. Eliminating the vasodilator effect of NO unmasks an underlying ET-1-mediated constriction.

Strong evidence exists for NOS III upregulation in the chronic hypoxia model of pulmonary hypertension (7, 9, 23, 27). In both acute and chronic hypoxic pulmonary hypertension, ET-1 is upregulated as well (2, 10). In the MCT model of pulmonary hypertension, recent studies documented enhanced NOS III localization (18) and increased NO production (25). In MCT-injected rats, ET-1 precursor mRNA is upregulated (13), and Matthew et al. (12) found elevated ET-1 levels in large pulmonary arteries. Although their data suggested that NOS may be downregulated because endothelium-dependent relaxation was inhibited, a study by Madden et al. (11) supported enhanced pulmonary NO production in MCT-injected rats. Our data support both NO (indirectly) and ET-1 (directly) upregulation.

Markedly elevated NO levels upregulate ET<sub>A</sub> receptors and enhance ET-1 affinity in cultured (systemic) smooth muscle cells (17). Others have also demonstrated functional interactions between ET-1 and NO in pulmonary vessels (29). Increasing evidence suggests that interactions among opposing vasoactive compounds may be involved in both normal and pathophysiological regulation of vascular tone.

The physiological significance of this dual upregulation is not known. Clearly, elevated endothelial NO production in this model serves to maintain active vasorelaxation and prevent the possibility of edema formation resulting from greatly elevated smooth muscle tone. Another possible function of this dual
upregulation is suggested by the hypoxic response data (Fig. 7). These data demonstrate that the modulating effect of NO on HPV is greater in pulmonary hypertensive compared with control rats. This could have a significant effect on the regulation of ventilation-to-perfusion ratios and consequently on active maintenance of gas-exchange efficiency.

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