Cirrhosis ameliorates monocrotaline-induced pulmonary hypertension in rats

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Short title: Monocrotaline and cirrhosis (28 characters)
Abstract (196 words)

Common bile duct ligation (CBDL) induces biliary cirrhosis, and pulmonary vasodilatation. We tested whether CBDL ameliorates monocrotaline (MCT)-induced pulmonary hypertension (PH) in rats.

Five groups of rats were studied: controls, MCT (60 mg/kg subcutaneously), CBDL, MCT followed by CBDL on day 7, and MCT plus CBDL (day 7) and L-NAME therapy between days 24 and 28. 28-day survival was 25% in the MCT group and 72% in the MCT+CBDL group. Pulmonary vascular resistance measured on days 21 and 28 increased in the MCT and MCT+CBDL+L-NAME groups but returned to normal in MCT+CBDL on day 28. Pulmonary artery (PA) medial hypertrophy persisted in MCT+CBDL. PA inflammation increased in MCT+CBDL with accumulation of both intra and peri-vascular macrophages. Exhaled NO decreased in the MCT group and increased in the MCT+CBDL group, which showed upregulation of inducible NO synthase and normal endothelial NO synthase. Blood endothelin (ET)-1 increased in CBDL, MCT, and MCT+CBDL. ET_B receptors increased and ET_A receptors decreased in the MCT+CBDL group, whereas the opposite changes occurred in the MCT group.

Biliary cirrhosis induces pulmonary vasodilation that ameliorates MCT-induced PH and improves survival. Upregulation of inducible NO synthase and ET_B receptor and downregulation of ET_A receptor may be involved.

**Key words:** Endothelin, Inflammation, Macrophages, Nitric oxide.
**Introduction**

Evidence is building that circulating macrophages recruited to the pulmonary circulation may play a major role in pulmonary hypertension (PH) due to various causes in humans and experimental animals (1). For example, inhibition of macrophage recruitment to the pulmonary arteries (2) halts the progression of monocrotaline (MCT)-induced PH in rats.

In humans and rats with liver cirrhosis, portosystemic shunts and impaired phagocytosis in the liver allow cytokines, circulating bacteria, and bacterial endotoxins from the gastrointestinal tract to enter the pulmonary circulation, where they damage the endothelium and induce extensive recruitment of macrophages to the arterial bed (3-8). These macrophages may play a critical role in the development of PH (7, 8) associated with liver cirrhosis (portopulmonary hypertension).

However, PH does not develop in cirrhotic rats and affects only a small minority of humans with cirrhosis. On the contrary, pulmonary vascular resistance is decreased, chiefly as a result of pulmonary vasodilation mediated mainly by nitric oxide (NO) (8). In rats with cirrhosis induced by common bile duct ligation (CBDL), NO is produced primarily by inducible NO synthase (iNOS) within pulmonary intravascular macrophages (8) and, to a lesser extent, by endothelial NO synthase (eNOS), which is activated by stimulation of the endothelin B (ET\textsubscript{B}) receptors (9). Thus, in liver cirrhosis, the pulmonary arteries (PA) are continuously exposed to mediators of inflammation but are usually protected against PH.

To further understand the complex interactions between liver injury and the development of PH, we investigated the pulmonary vascular response to liver cirrhosis induced by CBDL following MCT injection in rats. More specifically, we determined whether pulmonary vascular inflammation and changes in the endothelin and NO pathways induced by biliary cirrhosis ameliorated MCT-induced PH.
Methods

The study protocol was approved by our institutional animal care committee. Male Sprague-Dawley rats weighing 200–250 g were used. CBDL was performed 7 days after a single subcutaneous injection of MCT 60 mg/kg or vehicle. The rats were separated into five groups: MCT followed 7 days later by CBDL (MCT+CBDL group), MCT and sham operation (MCT group), vehicle injection and CBDL (CBDL group), and vehicle injection followed by a sham operation (control group). A last group of rats in the MCT+CBDL group received oral L-NAME (5 mg·kg⁻¹·d⁻¹) from day 24 to 28 (MCT+CBDL+L-NAME group). The rats were killed 28 days after MCT or vehicle injection (21 days after CBDL or sham operation).

Hemodynamic and blood gas measurements

Hemodynamic and blood gas values were recorded as previously described (5) on days 14, 21, and 28 after the injection (n=10 per group). Total pulmonary resistance index (TPRi) was calculated as mean pulmonary arterial pressure/cardiac index.

After the last hemodynamic measurement, laparotomy was performed. Blood from the aorta was used for measuring AST, ALT, and bilirubin. Mesenteric lymph nodes were cultured as previously described (6). The left lung was removed and frozen and the right lung was fixed in the distended state in formalin vehicle. The heart was dissected and weighed for calculation of the right ventricular (RV) hypertrophy index (ratio of RV over left ventricle + septum).

Exhaled NO was measured 15 minutes after each rat was sealed individually in a plastic box flushed with NO-free air, using a chemiluminescence NO analyzer (EVA 4000, Sérès, Aix en Provence, France). Exhaled NO concentration were measured before and 7, 14, 21, 25, 28 days after MCT or vehicle injection (n= 5 per group).
Plasma endothelin (ET)-1 concentrations were determined (n= 5 per group) using an ELISA kit (Santa Cruz Biotechnology, Santa Cruz, California) specific for rat ET-1 (10).

Protein expression of eNOS, iNOS, ETA and ETB receptors was determined in the lung using Western blotting (n= 5 per group). Protein were extracted from snap-frozen tissue samples (weight, 100mg) by homogenization in an appropriate amount of homogenizing buffer (Complete Mini, protease inhibitor cocktail [Roche Diagnostics, Mannheim, Germany] in phosphate-buffered saline [PBS] and 0.1% Triton X-100). The homogenates were centrifuged at 4°C and the supernatants were collected. After determination of the protein concentration, using the method of Bradford (10), 100 µg of protein from each lung sample was resuspended in 3X Laemmli buffer, boiled for 5 min, and separated on 10% acrylamide gels by electrophoresis. Proteins were electrophoretically transferred to a nitrocellulose membrane (Sigma-Aldrich) for 1 h at room temperature. After blocking with 5% bovine serum albumin in 1X Tween (T)–Tris buffered saline (TBS: 10 mM Tris-HCl [pH 8.0], 150 mM NaCl, and 0.1% Tween 20) for 2 h at room temperature, the membrane was washed three times with T–TBS at room temperature for 5 min. The membrane was incubated with anti-eNOS, iNOS, ETA receptor, and ETB receptor antibody (diluted 1:500; Transduction Laboratories, Lexington, UK and Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight with rocking. The membrane was then washed three times for 5 min each and incubated with secondary antibody conjugated with horseradish peroxidase, diluted 1:2,000, for 1-h at room temperature. Immunoreactive bands were detected with an enhanced chemiluminescence Western blotting analysis system (GE Healthcare UK, Little Chalfont, UK) and quantified by laser densitometry. Relative quantification was performed by normalization with β-actin (Sigma-Aldrich).

Lung immunohistochemistry.
Lung samples were fixed in 4% paraformaldehyde and paraffin-embedded tissues were sectioned at 4 μm. Lung staining was performed as described (11). After preparation and blocking, sections were incubated with ET<sub>B</sub> receptor (calbiochem-Novabiochem), or ED1 (Serotec) antibodies, washed and incubated with biotinylated secondary antibodies. After peroxydase-labeled streptavidin (Signet Laboratories, Dedham, MA) and diaminobenzidine (Biogenex, San Ramon, CA) development, sections were photographed using an axiophot microscope (Nikon, Melville, NY). The number of intravascular cells positive for the specific rat macrophage monoclonal antibody ED1 was determined in each vessel (external diameter inferior to 150 μm). Medial wall thickness of fully muscularized intraacinar arteries (50 to 100 μm in external diameter) expressed as a percentage of total vessel diameter was computed as [(external diameter-internal diameter)/external diameter]·100.

**Statistical Analysis**

Values are expressed as means±SEM. ANOVA was used where appropriate, with Fisher’s test for post hoc analysis. The survival rate was determined using Kaplan-Meier curves on day 28 after MCT injection in the MCT and MCT+CBDL groups, and the difference was evaluated using the log-rank test. P values less than 0.05 were considered significant.

**Results**

**Survival (Figure 1).** There were no deaths in the control or CBDL groups. Four-week survival was only 26% in the MCT group compared to 72% in the MCT+CBDL group (P<0.05). In these two groups, survival curves were nearly identical until day 20, after which survival dropped sharply in the MCT group.

**Hemodynamics, morphology, and blood gases (Table 1, Figure 1).** Pulmonary
Hypertension was noted on day 21 in the MCT+CBDL and MCT groups, with similar TPRi values (0.78±0.1 and 0.79±0.3 mm Hg·ml⁻¹·min⁻¹·100 g body weight, respectively). TPRi increased further in the MCT animals, which had severe PH on day 28 (mean PAP, 42±1.5 mm Hg) with a 4-fold increase in TPRi compared to the control group. By contrast, in the MCT+CBDL group, TPRi decreased to values similar to those in the control group on day 28. To determine whether TPRi normalization in the MCT+CBDL animals was caused by NO-mediated vasodilation, four MCT+CBDL rats were given the NOS inhibitor L-NAME from day 24 to day 28. In these rats, mean TPRi (0.90±0.04 mm Hg·ml⁻¹·min⁻¹·100 g body weight) was intermediate between the means in untreated MCT+CBDL rats (0.41±0.06 mm Hg·ml⁻¹·min⁻¹·100 g body weight) and in MCT rats (2.02±0.13 mm Hg·ml⁻¹·min⁻¹·100 g body weight), indicating that reversal of PH after CBDL was partly related to NO-mediated pulmonary vasodilation.

The CBDL and MCT+CBDL rats exhibited the hemodynamic pattern characteristic of hyperdynamic circulatory syndrome, with systemic arterial vasodilation manifesting as lower systemic vascular resistance values and higher cardiac index values, compared to the control and MCT animals.

RV hypertrophy was similar in the MCT and MCT+CBDL groups (RV hypertrophy index, 68%±10% and 58%±6%, respectively, NS difference).

The index of PA medial wall thickness was slightly lower in the MCT+CBDL group than in the MCT group (Table 1).

PaO₂ levels were lower in the CBDL group than in the other three groups (Table 1).

**Microbiology study.** Culture-positive mesenteric lymph nodes, indicating translocation, were found in 40% of MCT+CBDL rats and 20% of CBDL rats (non significant difference), and similar proportions of rats in these two groups had positive blood cultures. No bacterial translocation occurred in the MCT or control groups.
**Pulmonary macrophage sequestration.** All lungs from MCT+CBDL and CBDL rats showed accumulation of ED1-positive cells within the lumen of the PA (Figure 2). The number of intravascular ED1-positive cells per vessel was similar in these two groups (Figure 2). Numerous ED1-positive cells were found in the periadventitial space of the PA in the MCT and MCT+CBDL groups. No ED1-positive cells were seen in the lumen of PA of MCT rats, and neither were ED1-positive cells found in pulmonary vessels from control animals.

**Exhaled NO and NOS protein lung expressions (Figure 3).** Exhaled NO concentrations were lower in MCT than in control animals. MCT+CBDL rats had elevated exhaled NO values that were intermediate between those in the controls and in the CBDL rats. L-NAME reduced exhaled NO concentrations in MCT+CBDL+L-NAME rats to levels similar to those in MCT rats (not shown). The level of pulmonary lung eNOS protein expression was decreased in the MCT rats compared to the controls (Figure 3) \(P<0.01\). In contrast, no significant difference was found between pulmonary eNOS expression levels in MCT+CBDL and control rats. Pulmonary eNOS protein expression was higher in the CBDL rats than in the other three groups. Compared to the control and MCT groups, CBDL and MCT+CBDL groups (Figure 3) had higher levels of pulmonary iNOS protein expression. Pulmonary iNOS protein expression was higher in the MCT+CBDL group than in the CBDL rats. MCT and control rats had similar levels of pulmonary iNOS protein expression.

**ET-1 plasma and lung concentrations and ET\textsubscript{A} and ET\textsubscript{B} receptor protein expression in lungs (Figures 4 and 5).** Compared to the control group, the three other groups had higher plasma ET-1 concentrations, with no significant difference across these three groups. In the lungs, the balance of ET\textsubscript{A} to ET\textsubscript{B} receptors was altered, with decreased ET\textsubscript{A} receptor expression and increased ET\textsubscript{B} receptor expression in the MCT+CBDL group, contrasting with increased ET\textsubscript{A} receptor expression and decreased ET\textsubscript{B} expression in the MCT group (figure 4). Immunostaining
showed that increased levels of ET\(_B\) receptor in CBDL and MCT+CBDL lungs was consistent with an overexpression of this protein on pulmonary endothelium (Figure 5).

**Hepatic dysfunction.** CBDL induced jaundice in all rats and liver micronodularity with ascites in most, with no difference between the CBDL and MCT+CBDL groups. Gross and histological examination at autopsy showed cirrhosis in all rats subjected to CBDL (*i.e.* CBDL and MCT+CBDL groups). Compared to the CBDL group, higher values were found in the MCT+CBDL group for serum bilirubin (130±6 mmol/L and 212±77 mmol/L respectively), AST (94±20 UI/L and 270±105 UI/L respectively), and ALT (68±17 UI/L and 98±27 UI/L respectively). All these values were significantly higher than in the MCT and control groups: bilirubin (6±2 mmol/L and 8±3 mmol/L respectively), AST (94±21 UI/L and 102±24 UI/L respectively), and ALT (35±6 UI/L and 44±3 UI/L respectively).
Discussion

Monocrotaline administration to rats selectively injures the pulmonary vascular endothelium, inducing vasculitis with both perivascular infiltration of macrophages and increased proliferation and contraction of smooth muscle cells. The animals die about 28 days after MCT injection, due to a progressive increase in TPRi with subsequent right heart failure. Here, we demonstrated that liver cirrhosis induced by CBDL ameliorated MCT-induced PH and improved survival in MCT-exposed rats. Pulmonary vasodilation was the central mechanism of TPRi normalization, since pulmonary vascular remodeling was only slightly decreased. Pulmonary vasodilation was related in part to iNOS- driven NO overproduction, ET_{B} receptor upregulation, and ET_{A} receptor downregulation. Pulmonary vasculitis was characterized by accumulation of intravascular and extravascular macrophages. These results support our hypothesis that liver cirrhosis results in continuous exposure of the PA to mediators of inflammation but attenuates PH through activation of the endothelin and NO pathways.

To investigate the interactions between liver injury and the development of PH, we combined the effects of MCT exposure and biliary cirrhosis. CBDL was performed only one week after MCT injection, indicating that the amelioration of PH reflected the effects of liver cirrhosis on hypertensive pulmonary vessels, as opposed to abnormal hepatic metabolism of MCT. Liver injury was more severe in the MCT+CBDL rats than in the MCT rats or CBDL rats.

In keeping with earlier data (10), TPRi increased gradually until the third week after MCT injection, with no differences between groups with and without CBDL. By contrast, TPRi returned to normal during the fourth week in the MCT+CBDL group but continued to increase in the MCT group. The survival curves mirrored the TPRi changes, with a slight decrease in survival in both MCT-injected groups until day 21 and a sharp decline thereafter in the MCT group only (Figure 1). Survival rate was lower on day 28 after MCT injection in the MCT rats.
than in the MCT+CBDL rats, i.e., the group with the most severe degree of hepatic dysfunction. Altogether, these results indicate that pulmonary hemodynamic factors have a greater impact on survival rather than hepatic function in MCT+CBDL rats.

Although TPRi decreased rapidly to normal within one week (from days 21 to 28 after MCT administration), RV hypertrophy persisted on day 28 in the MCT+CBDL rats. These rats (and the CBDL rats) exhibited the hemodynamic features of hyperdynamic circulatory syndrome, with a 2-fold increase in pulmonary blood flow and a decrease in systemic vascular resistance. A reasonable hypothesis is that this pulmonary blood flow increase imposed an additional load on the RV, contributing to the persistent RV hypertrophy. This hypothesis is supported by recent studies (12) demonstrating that blood flow elevation worsened RV hypertrophy in MCT-exposed rats. Moreover, a longer period is required for reversal of RV hypertrophy after elimination of the pressure overload in MCT-induced PH.

Whereas TPRi returned to normal in the MCT+CBDL group, pulmonary vascular remodeling was only slightly diminished, indicating that the adverse effects of structural remodeling were overcome chiefly by pulmonary vasodilation. Because lung NO overproduction is the main factor in pulmonary vasodilation and hyperdynamic circulation in rats with biliary cirrhosis (8), we hypothesized that pulmonary vasodilation was mediated by NO in the MCT+CBDL rats. An interesting finding from our study is that PH was ameliorated between 14 and 21 days after CBDL, which is the period of maximally increased NO production (13). Exhaled NO concentrations 28 days after MCT injections were increased in the MCT+CBDL group (i.e., 21 days after CBDL) compared to the control group, whereas they were decreased in the MCT group. To further investigate the contribution of endogenous NO production to pulmonary vasodilation, the nonspecific NOS inhibitor L-NAME was administered orally to MCT+CBDL animals from day 24 to day 28 after MCT injection. As expected, L-NAME
increased TPRi to levels intermediate between those in untreated MCT+CBDL animals and MCT animals, and reduced exhaled NO concentrations to levels similar to those in MCT rats. This indicates that amelioration of PH was related in part to NO-mediated pulmonary vasodilation. To investigate which NOS isoforms were involved in pulmonary NO overproduction, we measured eNOS and iNOS protein expressions. In keeping with our previous studies in CBDL rats (3, 5, 6), we found increased iNOS expression in the lungs of MCT+CBDL rats but not of MCT rats. Lung eNOS protein expression remained within the normal range in the MCT+CBDL group but decreased in the MCT group, as previously reported (14). Thus, pulmonary NO overproduction was dependent on increased iNOS expression. Bacterial translocation (which occurred in CBDL and MCT+CBDL rats), endotoxins, and/or cytokines may have induced intravascular macrophage sequestration and iNOS upregulation in these macrophages, as previously reported in CBDL rats (3-7). Our finding that L-NAME incompletely inhibited pulmonary vasodilation suggests a role for the slight decrease in vascular remodeling and/or other vasodilators. Excessive release of carbon monoxide by intravascular macrophages or prostacyclin may be involved, since these vasodilators are upregulated in cirrhotic rats (15, 16) and are known to decrease TPRi in MCT-exposed rats (17, 18).

Our results regarding ET-1 and its receptors suggest an additional role for the endothelin pathway in the amelioration of MCT-induced PH in rats with biliary cirrhosis. ET-1 effects are mediated by activation of at least two receptor subtypes, ET_A and ET_B (19). ET_A receptors are located on smooth muscle cells and mediate vasoconstriction and smooth muscle proliferation (19). In contrast, ET_B receptors are found on both endothelial and smooth muscle cells in rats (19). Stimulation of endothelial ET_B receptors causes vasodilation through eNOS upregulation and prostacyclin release and also contributes to clear ET-1 from the circulation (19). Stimulation of ET_B receptors on smooth muscle causes vasoconstriction in the rat lung (19). Monocrotaline-
induced PH is characterized by elevated circulating ET-1 concentrations, downregulated lung ETB receptor, and upregulated lung ETA receptor (19, 20). Previous studies using specific ETB receptor antagonists or ETB receptor-deficient rats established a role for ET-1 in MCT-induced pulmonary hypertension involving amplification of ETA-mediated actions and lessening of ETB-protective effects (19, 20). Our findings suggest blunting or abolition of ET-1 effects, since MCT+CBDL rats had normal TPRi despite similar blood ET-1 elevation to that seen in MCT rats. The underlying process may involve regulation of the ET receptor balance. A previous study showed upregulation of pulmonary endothelial ETB receptor in CBDL lungs (9), which was responsible in part for pulmonary vasodilation via increases in NO production (by eNOS) and in prostacyclin synthesis. In our study, ETB receptor was upregulated in MCT+CBDL rats and downregulated in MCT rats. The upregulation in MCT+CBDL rats was associated with a significant increase of the ETB receptor expression on the pulmonary endothelium. This result is in agreement with a previous study demonstrating upregulation of pulmonary endothelial ETB receptor in CBDL lungs (9). Upregulation of ETB receptor is believed to increase eNOS expression (9, 21) and facilitate, by increasing intravascular macrophages recruitment, iNOS production (9). In keeping with those results, increased ETB receptor expression in our study may contribute to NO increased production (through eNOS and iNOS production). By contrast, ETA receptor expression increased in MCT rats and decreased in MCT+CBDL rats, consistent with previous studies in MCT rats (19) and CBDL rats (22). These opposite changes in the balance of ETA and ETB receptors in MCT+CBDL rats may play a protective role through inhibition of ET-1-induced vasoconstriction, prevention of eNOS downregulation, and iNOS upregulation.

One striking result in the MCT + CBDL group is the improvement of PaO2 compared to CBDL, suggesting that MCT pre-treatment may block the development of gas exchange abnormalities in CBDL animals. It is a substantial finding that exhaled NO is lower after MCT-
CBDL relative to CBDL, eNOS levels are low, there is a trend in the TPRi to increase and arterial PaO2 essentially normalizes relative to CBDL. This occurs despite the dramatic influx in macrophages and the high iNOS levels. These data suggest that endothelial injury induced by MCT may improve microvascular dilation after CBDL independent of the rise in intravascular macrophages. This finding is of great importance in understanding how the pulmonary vascular bed responds to the complex changes associated with biliary cirrhosis.

There are differences and similarities between our study and the recent study by Imamura et al. (22) on CDBL in rats exposed to chronic hypoxia. Cirrhotic rats exposed chronic hypoxia had normal TPRi values with a hyperdynamic circulatory state, similar changes in the balance of lung ET_A and ET_B receptors, and similar upregulation of lung NO production to those found in our animals. However, in contrast with our study, inhibition of pulmonary vascular remodeling was the central mechanism in the group exposed to chronic hypoxia. This discrepancy may result from the divergent effects of NO in pulmonary hypertension induced by hypoxia and MCT: NO treatment inhibits pulmonary vascular remodeling in hypoxia (23) but not MCT-induced PH (25). Chronic hypoxia and MCT are different models that induce distinct types of injury and patterns of gene expression (24). Pulmonary vascular remodeling and inflammation (including macrophage infiltration) are more severe with MCT than with chronic hypoxia (25). Thus, differences in the mechanisms of pulmonary vascular remodeling may explain the discrepancy in the results from these two studies.

Several limits of this study should be mentioned. First the protective role of cirrhosis on MCT-related PH attenuation was only studied according to a single sequence. Indeed, to ensure liver metabolism of MCT, the cirrhotic surgical procedure was performed after MCT subcutaneous injection. It could have been interesting to evaluate the same beneficial effect of
ciirrhosis if MCT were injected after the surgical procedure, which proved to be impractical. Second, this study individualized a distinctive distribution of macrophages in the different groups of the study: a perivascular accumulation of macrophages in the MCT groups and intravascular accumulation of macrophages in the cirrhotic group. These populations of macrophages may also play distinctive roles that need to be addressed in further studies.

In conclusion, this study demonstrates that development of biliary cirrhosis ameliorates MCT-induced PH and improves survival. Our data support the conclusion that, during biliary cirrhosis, increased amounts of iNOS-NO combined with selective upregulation of the ET$_B$ receptor and downregulation of the ET$_A$ receptor in the lungs lead to pulmonary vasodilation, with minimal changes in pulmonary vascular remodeling. The coexistence of pulmonary vascular remodeling and pulmonary vasodilation may result from recruitment of macrophages with opposite functional phenotypes to the pulmonary arterial system. Biliary cirrhosis induced recruitment of intravascular macrophages, leading to pulmonary vasodilation; whereas MCT induced recruitment of extravascular macrophages, leading to pulmonary vascular remodeling. These findings confirm our hypothesis that liver cirrhosis results in continuous exposure of the pulmonary arteries to mediators of inflammation but usually protects against PH.
References


Figure 1. Kaplan-Meier survival curves showing a significantly higher survival rate (top panel) and significantly lower total pulmonary resistance index values (bottom panel) in monocrotaline-exposed rats that underwent common bile duct ligation (MCT+CBDL, closed circles) compared to rats given MCT without CBDL (open circles). Values are means± SE; n=20 animals per group. Comparisons of TPRi: †: P<0.05 for day 21 compared to day 0, day 14 and day 28, *: P<0.05 for MCT compared to MCT+CBDL rats.
Figure 2. Micrographs showing positive staining of macrophages with the anti-rat macrophage antibody ED1 in the pulmonary artery of monocrotaline-exposed rats (MCT) with (left-hand top panel) or without (right-hand top panel) common bile duct ligation (CBDL). MCT rats had peri-arterial macrophages only, whereas MCT+CBDL rats had both intra- and peri-arterial macrophages. Medial hypertrophy is slightly less marked in the MCT+CBDL rat. Scale bar =100 µm. Number of pulmonary intravascular macrophages (PIMS) per vessel (bottom panel), identified as ED1-positive cells, in controls (vehicle injection and sham operation), rats injected with monocrotaline (MCT), rats injected with vehicle and subjected to common bile duct ligation (CBDL), and rats injected with MCT and subjected to CBDL (MCT+CBDL). In each animal, 60 vessels (external diameter < 150 µm) were examined, n=5 animals per group; values are means±SE. *: $P<0.05$, significant difference between the control and MCT groups.
Figure 3. Exhaled nitric oxide (NO) and lung relative protein level of inducible and endothelial NO synthases (iNOS and eNOS) in controls (vehicle injection and sham operation), rats injected with monocrotaline (MCT), rats injected with vehicle and subjected to common bile duct ligation (CBDL), and rats injected with monocrotaline and subjected to CBDL (MCT+CBDL). Values are means±SE; n=5 per group. *: *P<0.05, significantly different from control. †: †P<0.05, significantly different from MCT.

Figure 4. Plasma endothelin-1 (ET-1) concentration and lung relative protein level of ETB and ETA receptors in controls (vehicle injection and sham operation), rats injected with monocrotaline (MCT), rats injected with vehicle and subjected to common bile duct ligation
(CBDL), and rats injected with monocrotaline and subjected to CBDL (MCT+CBDL). Values are means±SE; n=5 per group. *: $P<0.05$, significantly different from control. †: $P<0.05$, significantly different from MCT.
Figure 5: Representative images of ET$_B$ receptor staining in medium-sized pulmonary arteries. In Control (A) and MCT (B) animals minimal ET$_B$ receptor staining was detectable along the endothelial surface. In CBDL (C) and MCT-CBDL (D) animals an increase in the ET$_B$ receptor signal was found in the intima of medium sized arterial vessels consistent with endothelial staining. No appreciable increase was observed elsewhere in the arterial wall. Staining was performed in two animals from each experimental group, and results are representative of the staining patterns observed in each animal. (original magnification, x 40).
Table 1. Hemodynamics, PaO2, right ventricular hypertrophy, and pulmonary artery remodeling in rats studied 28 days after monocrotaline injection.

<table>
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<th>Control</th>
<th>CBDL</th>
<th>MCT-CBDL</th>
<th>MCT</th>
<th>MCT+CBDL+L-NAME†</th>
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<td>PAP, mmHg</td>
<td>10 ± 0.3</td>
<td>9 ± 0.5†</td>
<td>16 ± 0.9†</td>
<td>42 ± 1.5*</td>
<td>24 ± 1.8*†</td>
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<td>CI, ml/min/100 g body wt</td>
<td>21 ± 1</td>
<td>30 ± 2*†</td>
<td>41 ± 5*†</td>
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<td>26 ± 3</td>
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<td>TPRi, mmHg.mL⁻¹.min⁻¹.100 g body wt</td>
<td>0.48 ± 0.03†</td>
<td>0.33 ± 0.03†</td>
<td>0.41 ± 0.06†</td>
<td>2.02 ± 0.13</td>
<td>0.91 ± 0.08†</td>
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<td>SVR, mmHg.ml⁻¹.min⁻¹.100 g body wt</td>
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<td>2.6 ± 0.2*†</td>
<td>2.5 ± 0.4*†</td>
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<td>PaO2, mmHg</td>
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<td>59 ± 3*</td>
<td>76 ± 1.5</td>
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<td>RV/(LV+septum)</td>
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<td>58 ± 6*</td>
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<td>15 ± 1</td>
<td>20 ± 2</td>
<td>44 ± 4*†</td>
<td>53 ± 6*</td>
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Values are means ± SE

CBDL: common bile duct ligation; MCT: monocrotaline; MCT+CBDL: monocrotaline injection followed 7 days later by common bile duct ligation; MCT+CBDL+L-NAME: monocrotaline injection followed 7 days later by common bile duct ligation and oral L-NAME (5 mg⁻¹·kg⁻¹·d⁻¹) from day 24 to 28; PAP: mean pulmonary arterial pressure; TPRi: total pulmonary resistance index, SVR: systemic vascular resistance; RV:
right ventricle; LV: left ventricle. *: P<0.05, significantly different from control. †: P<0.05, significantly different from MCT.

complementary group designed for hemodynamic evaluation only