Cirrhosis ameliorates monocrotaline-induced pulmonary hypertension in rats

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ABSTRACT: 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; rats dosed with MCT (60 mg·kg⁻¹ subcutaneously); CBDL; rats dosed with MCT followed by CBDL on day 7; and rats dosed with MCT followed by CBDL (day 7) and L-NAME therapy between days 24 and 28. 28-day survival was 26% 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 the MCT+CBDL group on day 28. Pulmonary artery (PA) medial hypertrophy persisted in MCT+CBDL rats. PA inflammation increased in MCT+CBDL rats, with accumulation of both intra- and perivascular macrophages. Exhaled nitric oxide (NO) levels 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 rats. Levels of 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.

KEYWORDS: Endothelin, inflammation, macrophages, nitric oxide

Evidence is growing that circulating macrophages recruited to the pulmonary circulation may play a major role in pulmonary hypertension (PH) of 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 (ET)_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 ET and NO pathways induced by biliary cirrhosis ameliorated MCT-induced PH.

METHODS

The study protocol was approved by our institutional animal care committee (Centre Chirurgial

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Marie Lannelongue, Université Paris Sud, Paris, France). 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⁻¹ 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). Finally, a group of rats in the MCT+CBDL group received oral L-NAME (5 mg·kg⁻¹·day⁻¹) on days 24–28 (MCT+CBDL+L-NAME group). The rats were killed 28 days after MCT or vehicle injection (21 days after CBDL or sham operation).

Haemodynamic and blood gas measurements

Haemodynamic 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 haemodynamic measurement, laparotomy was performed. Blood from the aorta was used for measuring aspartate aminotransferase (AST), alanine aminotransferase (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 to left ventricle+septum).

Exhaled NO was measured 15 min after each rat was sealed individually in a plastic box flushed with NO-free air, using a chemiluminescence NO analyser (EVA 4000; Sérès, Aix-en-Provence, France). Exhaled NO concentrations 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, CA, USA) specific for rat ET-1 [10].

Protein expression of eNOS, iNOS, ET_A and ET_B receptors was determined in the lung using Western blotting (n=5 per group). Protein was extracted from snap-frozen tissue samples (100 mg) by homogenisation in an appropriate amount of homogenising buffer (Complete Mini Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany) in 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 3 × Laemmli buffer, boiled for 5 min, and separated on 10% acrylamide gels by electrophoresis. Proteins were electrophoretically transferred to a nitrocellulose membrane (Sigma-Aldrich, St Louis, MO, USA) for 1 h at room temperature. After blocking with 5% bovine serum albumin in 1 × Tween–Tris-buffered saline (TBS: 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% Tween 20) for 2 h at room temperature, the membrane was washed three times with Tween–TBS at room temperature for 5 min. The membrane was incubated with anti-eNOS, -iNOS, -ET_A receptor, and -ET_B receptor antibodies (diluted 1:500; Transduction Laboratories, Lexington, UK and Santa Cruz Biotechnology, Santa Cruz, CA,

USA) 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 normalisation 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_B receptor (Calbiochem-Novabiochem, San Diego, CA, USA), or ED1 (Serotec, Kidlington, UK) antibodies, washed and incubated with biotinylated secondary antibodies. After peroxidase-labelled streptavidin (Signet Laboratories, Dedham, MA, USA) and diaminobenzidine (Biogenex, San Ramon, CA, USA) development, sections were photographed using an axiophot microscope (Nikon, Melville, NY, USA). The number of intravascular cells positive for the specific rat macrophage monoclonal antibody ED1 was determined in each vessel (external diameter <150 µm). Medial wall thickness of fully muscularised intra-acinar arteries (50–100 µm external diameter) expressed as a percentage of total vessel diameter was computed as follows:

$$\frac{(\text{external diameter} - \text{internal diameter})}{\text{external diameter}} \times 100$$

Statistical analysis

Values are expressed as mean ± 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 statistically significant.

RESULTS

Survival

There were no deaths in the control or CBDL groups. 4-week survival was only 26% in the MCT group compared with 72% in the MCT+CBDL group (fig. 1a; p<0.05). In these two groups, survival curves were nearly identical until day 20, after which survival dropped sharply in the MCT group.

Haemodynamics, morphology and blood gases

Pulmonary hypertension was noted on day 21 in the MCT+CBDL and MCT groups (fig. 1b), with similar TPRI values (0.78 ± 0.1 and 0.79 ± 0.3 mmHg·mL⁻¹·min⁻¹ (100 g body weight)), respectively). TPRI increased further in the MCT animals, which had severe PH on day 28 (mean pulmonary arterial pressure 42 ± 1.5 mmHg; table 1) with a four-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 normalisation in the MCT+CBDL animals was caused by NO-mediated vasodilation, four MCT+CBDL rats were given

FIGURE 1. a) Kaplan–Meier survival curve showing a significantly higher survival rate and b) significantly lower total pulmonary resistance index (TPRi) values in monocrotaline (MCT)-exposed rats that underwent common bile duct ligation (CBDL) (— and ●) compared with rats given MCT without CBDL (····· and ○). Data are presented as mean ± SEM; n=20 animals per group. #: p<0.05 for MCT versus MCT+CBDL rats; *: p<0.05 for day 21 compared with days 0, 14 and 28.

the NO synthase inhibitor L-NAME on days 24–28. In these rats, mean TPRi (0.90 ± 0.04 mmHg·mL⁻¹·min⁻¹·(100 g body weight)) was intermediate between the means in untreated MCT+CBDL rats (0.41 ± 0.06 mmHg·mL⁻¹·min⁻¹·(100 g body weight)) and in MCT rats (2.02 ± 0.13 mmHg·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 haemodynamic pattern characteristic of hyperdynamic circulatory syndrome, with systemic arterial vasodilation manifesting as lower systemic vascular resistance values and higher cardiac index values, compared with the control and MCT animals.

RV hypertrophy was similar in the MCT and MCT+CBDL groups (RV hypertrophy index $68\% \pm 10\%$ and $58\% \pm 6\%$, respectively, difference not statistically significant). The index

of PA medial wall thickness was slightly lower in the MCT+CBDL group than in the MCT group (table 1). Arterial oxygen tension (P_{a,O_2}) 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 (fig. 2). The number of intravascular ED1-positive cells per vessel was similar in these two groups (fig. 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 PA lumen of MCT rats, and nor were ED1-positive cells found in pulmonary vessels from control animals.

Exhaled NO and NO synthase protein expression in the lungs

Exhaled NO concentrations were lower in MCT than in control animals (fig. 3a). 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 (data not shown). Pulmonary eNOS protein expression was decreased in the MCT rats compared with the controls (fig. 3b) ($p<0.01$). 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 with the control and MCT groups, CBDL and MCT+CBDL groups (fig. 3c) 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_A and ET_B receptor protein expression in lungs

Compared with the control group, the three other groups had higher plasma ET-1 concentrations, with no significant difference across the three groups. In the lungs, the balance of ET_A to ET_B receptors was altered, with decreased ET_A receptor expression and increased ET_B receptor expression in the MCT+CBDL group, contrasting with increased ET_A receptor expression and decreased ET_B receptor expression in the MCT group (fig. 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 (fig. 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.*

TABLE 1 Haemodynamics, arterial oxygen tension (P_{a,O_2}), right ventricular hypertrophy and pulmonary artery remodelling in rats studied 28 days after monocrotaline (MCT) injection

	Control	CBDL	MCT+CBDL	MCT	MCT+CBDL+ L-NAME [#]
$\bar{P}_{pa}$ mmHg	10±0.3	9±0.5 [*]	16±0.9 ^{*,†}	42±1.5 [*]	24±1.8 ^{*,†}
CI mL·min ⁻¹ ·(100 g body weight) ⁻¹	21±1	30±2 ^{*,†}	41±5 ^{*,†}	21±1	26±3
TPRi mmHg·mL ⁻¹ ·min ⁻¹ ·(100 g body weight)	0.48±0.03 [†]	0.33±0.03 [†]	0.41±0.06 [†]	2.02±0.13	0.91±0.08 [†]
SVR mmHg·mL ⁻¹ ·min ⁻¹ ·(100 g body weight)	4.5±0.3	2.6±0.2 ^{*,†}	2.5±0.4 ^{*,†}	4.9±0.15	4.4±0.2
P_{a,O_2} mmHg	81±5	59±3 [*]	76±1.5	68±9	
RV/(LV+septum)	20±3	21±1	58±6 [*]	68±10 [*]	60±7 [*]
% Wall thickness	15±1	20±2	44±4 ^{*,†}	53±6 [*]	

Data are presented as mean±SEM. CBDL: common bile duct ligation; MCT+CBDL: MCT injection followed 7 days later by CBDL; MCT+CBDL+L-NAME: MCT injection followed 7 days later by CBDL and oral L-NAME (5 mg·kg⁻¹·day⁻¹) on days 24–28; $\bar{P}_{pa}$: mean pulmonary arterial pressure; CI: cardiac index; TPRi: total pulmonary resistance index; SVR: systemic vascular resistance; RV: right ventricle; LV: left ventricle. [#]: complementary group designed for haemodynamic evaluation only.

*: $p<0.05$ versus control; [†]: $p<0.05$ versus MCT.

CBDL and MCT+CBDL groups). Compared with the CBDL group, higher values were found in the MCT+CBDL group for serum bilirubin (130 ± 6 mmol·L⁻¹ and 212 ± 77 mmol·L⁻¹, respectively), AST (94 ± 20 UI·L⁻¹ and 270 ± 105 UI·L⁻¹, respectively) and ALT (68 ± 17 UI·L⁻¹ and 98 ± 27 UI·L⁻¹, respectively). All these values were significantly higher than in the MCT and control groups: bilirubin (6 ± 2 mmol·L⁻¹ and 8 ± 3 mmol·L⁻¹, respectively), AST (94 ± 21 UI·L⁻¹ and 102 ± 24 UI·L⁻¹, respectively) and ALT (35 ± 6 UI·L⁻¹ and 44 ± 3 UI·L⁻¹, respectively).

DISCUSSION

MCT 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, owing 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 normalisation, since pulmonary vascular remodelling 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 characterised 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 1 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 (fig. 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 haemodynamic factors have a greater impact on survival rather than hepatic function in MCT+CBDL rats.

Although TPRi decreased rapidly to normal within 1 week (from days 21–28 after MCT administration), RV hypertrophy persisted on day 28 in the MCT+CBDL rats. These rats (and the CBDL rats) exhibited the haemodynamic features of hyperdynamic circulatory syndrome, with a two-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 remodelling was only slightly diminished, indicating that the adverse effects of structural remodelling 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 hypothesised 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 with the control group, whereas

FIGURE 2. Micrographs showing positive staining of macrophages with the anti-rat macrophage antibody ED1 in the pulmonary artery of monocrotaline (MCT)-exposed rats with (a) or without (b) common bile duct ligation (CBDL). MCT rats had periarterial macrophages only, whereas MCT+CBDL rats had both intra- and periarterial macrophages. Medial hypertrophy is slightly less marked in the MCT+CBDL rat. Scale bars=100 µm. c) Number of pulmonary intravascular macrophages (PIMS) per vessel, identified as ED1-positive cells, in controls (vehicle injection and sham operation), rats injected with MCT, rats injected with vehicle and subjected to 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. Data are presented as mean±SEM. *: p<0.05 between the control and MCT groups.

they were decreased in the MCT group. To further investigate the contribution of endogenous NO production to pulmonary vasodilation, the nonspecific NO synthase inhibitor L-NAME was administered orally to MCT+CBDL animals from days 24–28 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 NO synthase isoforms were involved in pulmonary NO overproduction, we measured eNOS and iNOS protein expression. 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 in those 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 remodelling 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 ET 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 the clearance of ET-1 from the circulation [19]. Stimulation of ET_B receptors on smooth muscle causes vasoconstriction in the rat lung [19]. MCT-induced PH is characterised by elevated circulating ET-1 concentrations, downregulated lung ET_B receptor and upregulated lung ET_A

FIGURE 3. Exhaled nitric oxide (NO) and relative lung protein levels of endothelial and inducible NO synthases (eNOS and iNOS, respectively) 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). Data are presented as mean $\pm$ SEM. $n=5$ per group. *: $p<0.05$ versus control; #: $p<0.05$ versus MCT.

receptor [19, 20]. Previous studies using specific ET_B receptor antagonists or ET_B receptor-deficient rats established a role for ET-1 in MCT-induced pulmonary hypertension involving amplification of ET_A -mediated actions and lessening of ET_B -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 ET_B 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, the ET_B receptor was upregulated in MCT+CBDL rats and downregulated in MCT rats. The upregulation in MCT+CBDL rats was associated with a significant increase in ET_B receptor expression on the pulmonary endothelium. This result is in agreement with a previous study demonstrating upregulation of pulmonary endothelial ET_B receptor in CBDL lungs [9]. Upregulation of the ET_B receptor is believed to increase eNOS expression [9, 21] and to facilitate iNOS production, by increasing intravascular macrophage recruitment [9]. In keeping with those results,

increased ET_B receptor expression in our study may contribute to increased NO production (through eNOS and iNOS production). By contrast, ET_A 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 ET_A and ET_B 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 P_{a,O_2} compared with that in CBDL rats, 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 P_{a,O_2} essentially normalises 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

FIGURE 4. Plasma endothelin (ET)-1 concentration and lung relative protein level of endothelin ET_A and ET_B 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 MCT and subjected to CBDL (MCT+CBDL). Data are presented as mean $\pm$ SEM. $n=5$ per group. *: $p<0.05$ versus control; #: $p<0.05$ versus MCT.

FIGURE 5. Representative images of endothelin (ET)_B receptor staining in medium-sized pulmonary arteries. In a) control and b) monocrotaline (MCT)-treated animals, minimal ET_B receptor staining was detectable along the endothelial surface. In c) common bile duct ligation (CBDL) and d) MCT+CBDL animals, an increase in the ET_B receptor signal (arrows) 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. Scale bars=100 μm.

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 CBDL in rats exposed to chronic hypoxia. Cirrhotic rats exposed to 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 remodelling 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 remodelling in hypoxia [23] but not MCT-induced PH [24]. Chronic hypoxia and MCT are different models that induce distinct types of injury and patterns of gene expression [25]. Pulmonary vascular remodelling and inflammation (including macrophage infiltration) are more severe with MCT than with chronic hypoxia [24]. Thus, differences in the mechanisms of pulmonary vascular remodelling 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 subcutaneous MCT injection. It could have been interesting to evaluate the same beneficial effect of cirrhosis if MCT were injected after the surgical procedure, which proved to be impractical. Second, this study individualised 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 remodelling. The coexistence of pulmonary vascular remodelling 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 remodelling. 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.

SUPPORT STATEMENT

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STATEMENT OF INTEREST

Statements of interest for O. Sitbon and M. Humbert can be found at www.erj.ersjournals.com/misc/statements.dtl

REFERENCES

- Dorfmüller P, Perros F, Balabanian K, *et al.* Inflammation in pulmonary arterial hypertension. *Eur Respir J* 2003; 22: 358–363.
- Ikeda Y, Yonemitsu Y, Kataoka C, *et al.* Anti-monocyte chemoattractant protein-1 gene therapy attenuates pulmonary hypertension in rats. *Am J Physiol Heart Circ Physiol* 2002; 283: H2021–H2028.
- Rabiller A, Nunes H, Lebrec D, *et al.* Prevention of gram-negative translocation reduces the severity of hepatopulmonary syndrome. *Am J Respir Crit Care Med* 2002; 166: 514–517.
- Chang SW, Ohara N. Chronic biliary obstruction induces pulmonary intravascular phagocytosis and endotoxin sensitivity in rats. *J Clin Invest* 1994; 94: 2009–2019.
- Sztrymf B, Rabiller A, Nunes H, *et al.* Prevention of hepatopulmonary syndrome and hyperdynamic state by pentoxifylline in cirrhotic rats. *Eur Respir J* 2004; 23: 752–758.
- Sztrymf B, Libert JM, Mougeot C, *et al.* Cirrhotic rats with bacterial translocation have higher incidence and severity of hepatopulmonary syndrome. *J Gastroenterol Hepatol* 2005; 20: 1538–1544.
- Rodriguez-Roisin R, Krowka MJ, Herve P, *et al.* Pulmonary–hepatic vascular disorders (phd). *Eur Respir J* 2004; 24: 861–880.
- Nunes H, Lebrec D, Mazmanian M, *et al.* Role of nitric oxide in hepatopulmonary syndrome in cirrhotic rats. *Am J Respir Crit Care Med* 2001; 164: 879–885.
- Ling Y, Zhang J, Luo B, *et al.* The role of endothelin-1 and the endothelin B receptor in the pathogenesis of hepatopulmonary syndrome in the rat. *Hepatology* 2004; 39: 1593–1602.
- Guignabert C, Raffeston B, Benferhat R, *et al.* Serotonin transporter inhibition prevents and reverses monocrotaline-induced pulmonary hypertension in rats. *Circulation* 2005; 111: 2812–2819.
- Myers RB, Oelschlager D, Manne U, *et al.* Androgenic regulation of growth factor and growth factor receptor expression in the CWR22 model of prostatic adenocarcinoma. *Int J Cancer* 1999; 82: 424–429.
- van Albada ME, Schoemaker RG, Kemna MS, *et al.* The role of increased pulmonary blood flow in pulmonary arterial hypertension. *Eur Respir J* 2005; 26: 487–493.
- Zhang J, Ling Y, Luo B, *et al.* Analysis of pulmonary heme oxygenase-1 and nitric oxide synthase alterations in experimental hepatopulmonary syndrome. *Gastroenterology* 2003; 125: 1441–1451.
- Kanno S, Wu YJ, Lee PC, *et al.* Angiotensin-converting enzyme inhibitor preserves p21 and endothelial nitric oxide synthase expression in monocrotaline-induced pulmonary arterial hypertension in rats. *Circulation* 2001; 104: 945–950.
- Luo B, Liu L, Tang L, *et al.* ET-1 and TNF- α in HPS: analysis in prehepatic portal hypertension and biliary and nonbiliary cirrhosis in rats. *Am J Physiol Gastrointest Liver Physiol* 2004; 286: G294–G303.
- Colle IO, De Vriese AS, Van Vlierberghe HR, *et al.* Vascular hyporesponsiveness in the mesenteric artery of anesthetized rats with cirrhosis and portal hypertension: An *in-vivo* study. *Eur J Gastroenterol Hepatol* 2004; 16: 139–145.
- Goto J, Ishikawa K, Kawamura K, *et al.* Heme oxygenase-1 reduces murine monocrotaline-induced pulmonary inflammatory responses and resultant right ventricular overload. *Antioxid Redox Signal* 2002; 4: 563–568.
- Hill LL, Pearl RG. Combined inhaled nitric oxide and inhaled prostacyclin during experimental chronic pulmonary hypertension. *J Appl Physiol* 1999; 86: 1160–1164.
- Michel RP, Langleben D, Dupuis J. The endothelin system in pulmonary hypertension. *Can J Physiol Pharmacol* 2003; 81: 542–554.
- Nishida M, Eshiro K, Okada Y, *et al.* Roles of endothelin ETA and ETB receptors in the pathogenesis of monocrotaline-induced pulmonary hypertension. *J Cardiovasc Pharmacol* 2004; 44: 187–191.
- Tang L, Luo B, Patel RP, *et al.* Modulation of pulmonary endothelial endothelin B receptor expression and signaling: implications for experimental hepatopulmonary syndrome. *Am J Physiol Lung Cell Mol Physiol* 2007; 292: L1467–L1472.
- Imamura M, Luo B, Limbird J, *et al.* Hypoxic pulmonary hypertension is prevented in rats with common bile duct ligation. *J Appl Physiol* 2005; 98: 739–747.
- Horstman DJ, Frank DU, Rich GF. Prolonged inhaled NO attenuates hypoxic, but not monocrotaline-induced, pulmonary vascular remodeling in rats. *Anesth Analg* 1998; 86: 74–81.

- 24** van Suylen RJ, Smits JF, Daemen MJ. Pulmonary artery remodeling differs in hypoxia- and monocrotaline-induced pulmonary hypertension. *Am J Respir Crit Care Med* 1998; 157: 1423–1428.
- 25** Partovian C, Adnot S, Eddahibi S, *et al.* Heart and lung VEGF mRNA expression in rats with monocrotaline- or hypoxia-induced pulmonary hypertension. *Am J Physiol* 1998; 275: H1948–H1956.