Reduction in hepatic endothelin-1 clearance in cirrhosis

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ABSTRACT

Circulating endothelin-1 (ET-1) levels are increased in cirrhosis. The liver is an important site for circulating ET-1 clearance through the ETB receptor. We evaluated ET-1 kinetics in cirrhosis to determine if a reduced liver clearance contributes to this process. Cirrhosis was induced by carbon tetrachloride in rats. Hepatic ET-1 clearance was measured in isolated perfused livers using the single bolus multiple indicator-dilution technique. Plasma ET-1 levels doubled in cirrhosis from 0.49 ± 0.04 fmol/ml (mean ± S.E.M.) to 1.0 ± 0.18 fmol/ml (P < 0.01). Liver ET-1 extraction was reduced from 81 ± 1% (mean ± S.E.M.) in controls to 50 ± 6% in cirrhosis (P < 0.01). Kinetic modelling revealed a major irreversible binding site for ET-1 that is blocked by the selective ETB receptor antagonist BQ788 and a minor non-specific reversible binding site that cannot be blocked with BQ788 or the selective ETA antagonist BQ123. Reduced hepatic clearance correlated with the biochemical markers of cirrhosis, portal vein perfusion pressure (r = −0.457; P < 0.001) and the increase in ET-1 levels (r = −0.462; P = 0.002). Immunohistofluorescence with specific anti-(ETB receptor) antibodies revealed a preponderant distribution of ETB receptors on hepatic stellate cells, which was increased in cirrhosis. We conclude that cirrhosis reduces ET-1 clearance probably by capillarization of hepatic sinusoids and reduced access to ETB receptors. This relates to the severity of cirrhosis and may contribute to the increase in circulating ET-1 levels.

INTRODUCTION

Endothelin-1 (ET-1) is a potent vasoconstrictive and promitogenic peptide. In liver cirrhosis, a majority of investigators have reported increased plasma ET-1 levels [1–5]. Compared with control subjects, patients with cirrhosis demonstrate a net hepatosplanchnic release of ET-1, suggesting that the liver may in part contribute to this increase [6,7]. The mechanisms responsible for circulating ET-1 elevation in cirrhosis and its pathophysiological role, however, remain poorly defined.

The liver is an important site for circulating ET-1 clearance with close to 85% extraction in the dog liver in vivo [8]. This clearance is mediated by the ETB receptor [8]. Higher hepatic vein ET-1 levels by comparison with portal vein levels has led investigators to postulate that the cirrhotic human liver produces and releases ET-1 into circulation [9]. The finding of increased hepatic tissue ET-1 levels that correlate with disease severity strengthens this hypothesis [10]. Consequently, an increase in hepatic vein ET-1 levels in cirrhosis may result not only from increased ET-1 production and release by the liver, but also from reduced ET-1 clearance, or from a combination of both; measurements of ET levels across an organ with simultaneous production and clearance does not, however, allow these distinctions. The primary aim of this

Key words: endothelium, indicator-dilution technique, mathematical modelling, peptide, pharmacology.

Abbreviations: CLpi, clearance by the irreversible binding sites; CLpr, clearance by the reversible binding sites; ET-1, endothelin-1; i.p., intraperitoneal; kpi, transfer co-efficient for binding from plasma to irreversible binding sites; kp,r, transfer co-efficient for binding from plasma to reversible binding sites; kpi,p, transfer co-efficient for dissociation from the reversible binding sites to plasma.

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study was therefore to quantify and characterize hepatic ET-1 clearance kinetics in cirrhosis in order to determine if reduced clearance could contribute to this process.

**MATERIALS AND METHODS**

**Experimental groups**

The study protocol was approved by the Animal Research Committee of the Montreal Heart Institute and conducted according to the Canadian Guidelines for the Care of Laboratory Animals. Cirrhosis ($n = 16$) was induced in male Sprague–Dawley rats weighing between 150–200 g by intraperitoneal (i.p.) injections of 0.2 ml of carbon tetrachloride [1:6 (v/v) in peanut oil] twice a week for 12 weeks. Sodium phenobarbital (0.4 g/l) was added to drinking water 3 days before the first dose of carbon tetrachloride and continued for the 12 weeks. The control group ($n = 12$) received i.p. injections of 0.2 ml of peanut oil only according to the same regimen without addition of sodium phenobarbital in the drinking water. To determine any effect of the hepatic enzyme inducer alone, a third group (phenobarbital group; $n = 17$) received i.p. injections of 0.2 ml of peanut oil only with the addition of sodium phenobarbital (0.4 g/l) in drinking water according to the same regimen. All study drugs were stopped 1 week before studies.

**Plasma samples and liver isolation**

The animals were anaesthetized with halothane, intubated and mechanically ventilated. Blood samples were collected from the right jugular vein to determine biochemical liver function tests and plasma ET-1 levels using a commercially available ELISA kit (Biomedica).

After a median laparotomy, the portal vein was isolated and cannulated with a 16-gauge blunt needle. The outflow cannula was inserted into the inferior vena cava through an incision into the right atrium. The liver was then perfused *in situ* at a constant rate of 20 ml/min at 37 °C with a Krebs solution (120 mM NaCl, 25 mM NaHCO$_3$, 4.7 mM KCl, 1.18 mM KH$_2$PO$_4$, 1.17 mM MgSO$_4$, 2.5 mM CaCl$_2$ and 5.5 mM glucose) supplemented with 3% (w/v) human albumin and with a pH adjusted to 7.4. The solution was oxygenated with a mixture of 95% O$_2$ and 5% CO$_2$ and perfused in a non-recirculating fashion. The kinetic parameters optimized by the model are: $k_{pi}$, transfer co-efficient for binding from plasma to irreversible binding sites; $k_{pr}$, transfer co-efficient for binding from plasma to reversible binding sites; and $k_{pn}$, transfer co-efficient for dissociation from reversible binding sites to plasma. The corresponding clearances for the irreversible ($CL_{pi}$) and reversible ($CL_{pr}$) binding sites were also computed as described previously [8].

**Indicator-dilution study**

An indicator-dilution experiment was performed by rapidly injecting 0.2 ml of tracers into the portal vein and simultaneously collecting timed outflow samples (40 tubes in 40 s). The injectate was prepared by adding 0.5 µCi of $^{125}$I-labelled ET-1 (NEN Life Science Products Inc.) to 2.0 ml of 0.9% saline and 0.5 ml of Evans Blue dye (5 mg/ml). BSA (Fisher Scientific) was added to a concentration of 4 g/100 ml. The blue dye binds tightly to albumin, which can then be used as a vascular reference tracer. A portion (0.2 ml) of the mixture was used as the indicator-dilution curve bolus, whereas the remainder was kept to prepare standards. The collected tubes and standards for each experiment were processed identically in order to determine the activity of each tracer. Saline (2 ml of 0.9% saline) was added into each tube and vortex-mixed. A portion (1 ml) of this solution was pipetted into a spectrophotometer cuvette to determine the absorbance of the Evans Blue dye. The remainder was placed in a γ-counter to determine $^{125}$I radioactivity. The fractional recovery of each tracer in each sample was then determined to construct concentration versus time curves in the usual fashion. In order to calculate the proportion of $^{125}$I-labelled ET-1 surviving passage through the liver (recovery), areas under the curves were calculated using power law extrapolation [11].

**ET-1 recovery and kinetic modelling**

The model we have developed previously to describe ET-1 kinetics in the dog liver was used [8]. This barrier-limited space-distributed variable-transit-time model describes the microcirculatory exchanges of ET-1 between liver cell and plasma by relating the outflow profile for the substance under study, $^{125}$I-labelled ET-1, and that of a reference substance, $^{125}$I-labelled albumin, which is not taken up by the cells, but behaves similarly in all other respects. The model incorporates the non-limiting binding of ET-1 to albumin and provides optimal fits when including two distinct binding sites for ET-1 on liver cells: a predominant irreversible binding site and a minor reversible binding site. The same model was thus applied using $^{125}$I-labelled albumin as a vascular reference and provided fits of excellent quality. A schematic representation of the model is presented in Figure 1. The kinetic parameters optimized by the model are: $k_{pi}$, transfer co-efficient for binding from plasma to irreversible binding sites; $k_{pr}$, transfer co-efficient for binding from plasma to reversible binding sites; and $k_{pn}$, transfer co-efficient for dissociation from reversible binding sites to plasma. The corresponding clearances for the irreversible ($CL_{pi}$) and reversible ($CL_{pr}$) binding sites were also computed as described previously [8].

**Effects of ET receptor antagonists on ET-1 kinetics in the rat liver**

To determine the modulatory roles of ET receptors in rat liver ET-1 clearance, we performed additional experiments. Rat livers were isolated as above and a baseline indicator-dilution curve was performed. The selective ET$_A$ receptor antagonist BQ788 ($n = 4$) or selective ET$_A$ receptor antagonist BQ123 ($n = 4$) were then perfused at a concentration of $10^{-4}$ mol/l for 15 min, after which a second indicator-dilution experiment was performed.
Figure 1  Schematic representation of the model used to compute ET-1 kinetics in the isolated rat liver

$k_{pr}$, transfer co-efficient for binding from plasma to reversible binding sites; $k_{rp}$, transfer co-efficient for dissociation from the reversible binding sites to plasma; and $k_{pi}$, transfer co-efficient for binding from plasma to irreversible binding sites.

**Histological analysis**

Rat livers were cut into slices of 3 mm thickness and fixed overnight in 10 % buffered formalin (Sigma). Sections of 5 μm were stained with Masson’s trichrome solution for collagen staining, after an appropriate dehydration and paraffin embedding. All slices were examined with a microscope (Olympus BX-60, Olympus America Inc.) using a 4× objective (40× magnification).

**Immunohistofluorescence analyses**

Liver sections (10 μm) were fixed with acetone, permeabilized with 0.1 % saponin and blocked with 10 % (v/v) normal donkey serum in PBS. A rabbit anti-(ET$_B$ receptor) antibody (Alomone Labs) diluted with 2 % (v/v) normal donkey serum (1:100) was applied and incubated for 1 h at room temperature and then overnight at 4 °C. After washing, the sections were incubated for 1 h at room temperature with a donkey anti-(rabbit IgG) antibody (Jackson Immunoresearch Inc.) conjugated with TRITC (tetramethylrhodamine β-isothiocyanate) at a 1:400 dilution in 2 % (v/v) normal donkey serum in PBS. After washing, the sections were mounted and examined with a Zeiss Axiovert microscope equipped with the LSM 510 confocal imaging system. To determine non-specific binding, control experiments with secondary antibody without primary antibody were also performed.

**Statistical analysis**

All values are expressed as means ± S.E.M. Differences between the control, phenobarbital and cirrhosis groups were evaluated by ANOVA, followed by multiple group comparisons using the Bonferroni correction. The effects of the ET receptor antagonists were evaluated by two-tailed paired $t$ tests. $P < 0.05$ was considered significant.

**RESULTS**

The cirrhosis group had macroscopic evidence of liver cirrhosis confirmed by the histological presence of fibrosis and regenerescence nodules (Figure 2). Morphological and biochemical parameters as well as isolated liver portal vein perfusion pressures are shown in Table 1.
Table 1  Morphological and biochemical parameters

*P < 0.05 and †P < 0.01 compared with the control group; §P < 0.01 compared with the phenobarbital group.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (n = 12)</th>
<th>Phenobarbital (n = 17)</th>
<th>Cirrhosis (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Morphological parameters</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>532 ± 15</td>
<td>526 ± 12</td>
<td>444 ± 16 §</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>15.5 ± 0.6</td>
<td>16.1 ± 0.5</td>
<td>15.9 ± 0.9</td>
</tr>
<tr>
<td>Liver/body weight</td>
<td>0.027 ± 0.000</td>
<td>0.031 ± 0.001 §</td>
<td>0.036 ± 0.001 † §</td>
</tr>
<tr>
<td><strong>Biochemical parameters</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine aminotransferase (units/l)</td>
<td>43 ± 3</td>
<td>44 ± 6</td>
<td>94 ± 8 §</td>
</tr>
<tr>
<td>Total bilirubin (µmol/l)</td>
<td>1.4 ± 0.3</td>
<td>1.2 ± 0.1</td>
<td>5.2 ± 11 † §</td>
</tr>
<tr>
<td>Alkaline phosphatase (units/l)</td>
<td>135 ± 11</td>
<td>102 ± 8</td>
<td>297 ± 20 † §</td>
</tr>
<tr>
<td>Albumin (g/l)</td>
<td>34 ± 1.0</td>
<td>34 ± 0.6</td>
<td>29 ± 1.4 † §</td>
</tr>
<tr>
<td>Creatinine (µmol/l)</td>
<td>44 ± 3.6</td>
<td>41 ± 1.9</td>
<td>44 ± 9.9</td>
</tr>
<tr>
<td>Portal vein perfusion pressure (mmHg)</td>
<td>7.3 ± 0.4</td>
<td>8.7 ± 0.3</td>
<td>10.8 ± 0.7 † §</td>
</tr>
</tbody>
</table>

Body weight was similar at baseline (results not shown) and was reduced after 12 weeks in the cirrhotic animals. There was no difference in absolute liver weight, but the smaller body weight resulted in a higher liver/body weight ratio in the cirrhotic animals. Alanine aminotransferase, total bilirubin and alkaline phosphatase were all elevated in the cirrhosis group. Plasma albumin was reduced by cirrhosis, whereas creatinine was unaffected. There was an approx. 45 % increase in portal perfusion pressure in the cirrhotic animals compared with the controls.

Plasma ET-1 levels doubled in cirrhosis (Figure 3). Liver ET-1 extraction was 81 ± 1 % in the control group (Figure 4). This was slightly reduced in the phenobarbital group to 69 ± 3 % (P = 0.032) and markedly reduced in the cirrhosis group to 50 ± 6 % (P < 0.01). The kinetic parameters for hepatic ET-1 metabolism are presented in Table 2. The rate constants for binding to and unbinding from the reversible site (kₚᵣ and kᵣₚ) were reduced only by cirrhosis. Cirrhosis reduced plasma clearance of ET-1 by both the irreversible and the reversible binding sites, whereas only clearance by the irreversible site was reduced after phenobarbital administration alone.

The reduction in ET-1 extraction correlated with levels of alanine aminotransferase (r = −0.489; P = 0.002), total bilirubin (r = −0.641; P < 0.001), alkaline phosphatase (r = −0.551; P < 0.001), portal vein perfusion pressure (r = −0.457; P < 0.001), albumin (r = 0.502; P < 0.001), central venous pressure (r = −0.489; P < 0.001) and plasma ET-1 levels (r = −0.462; P = 0.002) (Figure 5). The selective ETA receptor antagonist BQ123 did not affect ET-1 extraction or the model-derived kinetic
Table 2  Model-optimized ET-1 kinetic parameters in the liver

<table>
<thead>
<tr>
<th>Groups</th>
<th>kinetic parameters</th>
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<tbody>
<tr>
<td></td>
<td>$k_{p1}$ (s$^{-1}$)</td>
<td>$k_{p2}$ (s$^{-1}$)</td>
</tr>
<tr>
<td>Control ($n=12$)</td>
<td>0.347±0.035</td>
<td>0.174±0.048</td>
</tr>
<tr>
<td>Phenobarbital ($n=17$)</td>
<td>0.221±0.019†</td>
<td>0.134±0.013</td>
</tr>
<tr>
<td>Cirrhosis ($n=16$)</td>
<td>0.163±0.028†</td>
<td>0.057±0.010*</td>
</tr>
<tr>
<td>BQ123 ($n=4$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-antagonist</td>
<td>0.360±0.130</td>
<td>0.148±0.059</td>
</tr>
<tr>
<td>Post-antagonist</td>
<td>0.217±0.021</td>
<td>0.093±0.026</td>
</tr>
<tr>
<td>BQ788 ($n=4$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-antagonist</td>
<td>0.381±0.067</td>
<td>0.103±0.040</td>
</tr>
<tr>
<td>Post-antagonist</td>
<td>0.075±0.017§</td>
<td>0.036±0.017</td>
</tr>
</tbody>
</table>

The selective ET$_B$ receptor antagonist, however, markedly reduced ET-1 extraction from 82±5% to 53±6% ($P<0.01$). This was associated with a reduction in the rate constant for binding to the irreversible site as well as in clearance by this site (Table 2). Clearance from the reversible binding site was slightly, but significantly, reduced as well by the ET$_B$ receptor antagonist.

Confocal microscopy of the ET$_B$ receptor demonstrated staining of cells possessing multiple projections within the sinusoidal space (Figure 7). The location and morphology of these cells was typical of hepatic stellate cells. Some staining was found at the lining of the sinusoidal space, but was essentially absent from the hepatocytes. In the cirrhotic animals, there was more abundant and intense staining; the stellate cells were more numerous with bigger and longer cytoplasmic projections (Figure 7). The phenobarbital group did not demonstrate appreciable differences in staining compared with controls (results not shown). There was no detectable staining of sections that were not treated with the primary antibody.

DISCUSSION

The liver as a major site for ET-1 clearance

The pulmonary circulation is recognized as the most important site for circulating ET-1 clearance with approx. 50% single-pass ET-1 extraction in humans [12] and 40% in dogs [13]. Single-pass ET-1 extraction in the dog liver in vivo (85%) [8] is more than double that...
in the lungs, but net clearance (the product of flow and extraction) will be lower, since the liver receives less than one-third of the whole cardiac output. In the present study in isolated rat livers, we found a similar mean single-pass ET-1 extraction (83%), which is higher than that for isolated rat lungs (60%) [14]. Intravenously injected $^{125}$I-labelled ET-1 has a half-life of less than 7 min in rats, with most of the label retained by the lungs, followed in importance by the kidney and the liver [15]. Our findings are compatible with these experiments and firmly establish the importance of the liver as a clearance site for circulating ET-1.

**ET-1 kinetics in the rat liver**

The kinetic modelling applied to the data predicts the presence of a major irreversible binding site and a minor reversible binding site. Binding to the irreversible site is modulated by a selective ET$_B$ receptor antagonist, whereas binding to the reversible site is not notably affected by either ET$_A$ or ET$_B$ receptor antagonism.

Immunohistochemical analysis of normal rat and human livers reveals the presence ET$_B$ receptors on hepatic sinusoidal lining cells which, by immunogold electron microscopic analysis, were identified as hepatic stellate cells and sinusoidal endothelial cells [16,17]. Since we

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**Figure 7** Immunohistofluorescence staining of the ET$_B$ receptor in the control (A and C) and the cirrhotic (B and D) rat liver

(A and B) The aggregate of slices through a 10 $\mu$m thick section. There is more diffuse and intense staining (light areas) in cirrhosis. (C) A single slice of a normal section with staining of cells characteristic of hepatic stellate cells. (D) A higher-power view of a densely stained hepatic stellate cell in cirrhosis. Magnification, $\times$40 (A–C) and $\times$63 (D).
demonstrate that ET-1 removal is ET\textsubscript{B}-receptor-dependent, access into the Disse space, where hepatic stellate cells and sinusoidal endothelial cells reside, must be a prerequisite for circulating ET-1 clearance.

Reduced ET-1 clearance in cirrhosis

ET-1 extraction was markedly reduced in relation to the severity of cirrhosis. It also correlated with the increase in circulating ET-1 levels. Our present data therefore demonstrate for the first time that a reduced hepatic ET-1 clearance can contribute to the observed increase in circulating ET-1 levels. It is probable, however, that other mechanisms not addressed in the present study could also contribute to the process, such as liver and/or splanchnic release of ET-1 as well as imbalance in the production and clearance of ET-1 by other organs. Since only a small fraction of the secreted ET-1 is normally released into plasma, the pathophysiological significance of increased plasma ET-1 levels in cirrhosis is currently unknown.

Rate constants for binding to both the irreversible as well as the reversible sites were reduced by cirrhosis. The clearances mediated by these sites were consequently reduced by approx. 50\% (Table 2) compared with controls. To gain more insight into the mechanisms responsible for this reduced clearance, we qualitatively assessed the distribution of the ET\textsubscript{B} receptor in the liver using immunohistofluorescence. This approach revealed predominant staining of hepatic stellate cells. In cirrhosis, staining became more intense and diffuse, with larger stellate cells possessing more developed projections. These findings are in complete agreement with those of Yokomori et al. [16,17] in the same model [16] as well as in human cirrhosis [17]. In both preparations, these investigators [16,17] demonstrated increased ET\textsubscript{B} receptor staining on hepatic stellate cells and sinusoidal endothelial cells, particularly on hepatic stellate cells. In the carbon tetrachloride model of rat cirrhosis, Ghandi et al. [18] have shown an increase in total ET receptor density with no change in affinity, whereas, in the biliary liver fibrosis model, another study [19] has shown a 5-fold increase in ET\textsubscript{B} receptor density with a significant reduction in binding affinity.

The probable mechanism for the reduced ET-1 clearance in cirrhosis must therefore be decreased access to the binding sites secondary to fibrosis and capillarization of hepatic sinusoids, a process which is related to the severity of cirrhosis.

The hepatic enzyme inducer sodium phenobarbital caused a mild significant increase in the liver/body weight ratio and a non-significant increase in portal perfusion pressure. The phenobarbital group also demonstrated a small, but significant, decrease in liver ET-1 extraction. Kinetic modelling revealed that this was caused by a reduction in the rate constant for binding to the irreversible site with a resultant significant reduction in clearance by this site. Although speculative, it is possible that phenobarbital administration results in down-regulation or desensitization of liver ET\textsubscript{B} receptors. The immunofluorescence analysis we have performed, however, did not suggest reduced ET\textsubscript{B} receptor density in the phenobarbital group. The small effect of phenobarbital alone on ET-1 kinetics confirms that our findings were secondary to the induction of cirrhosis.

Conclusion

The liver is an important site for circulating ET-1 clearance, which is mediated by the ET\textsubscript{B} receptor present on hepatic sinusoidal cells, mostly hepatic stellate cells. Cirrhosis causes a reduction in liver ET-1 clearance in relation to the severity of the disease. The reduced clearance probably relates to a reduced access of circulating ET-1 to the Disse space secondary to capillarization of sinusoids and may contribute to the observed increase in circulating ET-1 levels in cirrhosis.

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