



Paracrine renal endothelin system in rats with liver cirrhosis

^{1,*}†Berthold Hoher, †Rüdiger Zart, *Fritz Diekmann, ** Peter Rohmeiss, #Armin Distler, *Hans H. Neumayer, †Christian Bauer & #Peter Gross

Departments of Nephrology, *Universitätsklinikum Charité der Humboldt Universität zu Berlin, **Klinikum Mannheim, University of Heidelberg and #Universitätsklinikum Benjamin Franklin, Free University of Berlin, and †Institute of Molecular Biology and Biochemistry, Free University of Berlin, Germany

1 Liver cirrhosis was induced in rats by CCl₄ administration. We analysed the expression of endothelin receptor subtypes in the renal cortex and medulla using Scatchard analysis and receptor autoradiography, and measured plasma as well as renal-tissue endothelin-1 concentrations using a specific radioimmunoassay. Furthermore, we analysed the effects of the non-selective (A/B) endothelin receptor antagonist, bosentan (6 and 100 mg kg⁻¹ day⁻¹) on mean arterial blood pressure, water and sodium excretion and glomerular filtration rate.

2 Our study revealed an overexpression of the endothelin B receptor (ET_B) in the renal medulla of rats with liver cirrhosis (Cir: 2775 ± 299 fmol mg⁻¹; Con: 1695 ± 255 fmol mg⁻¹; *n* = 8; means ± s.d., *P* < 0.01), whereas the density of ET_B in the cortex and the endothelin A receptor (ET_A) in the cortex and medulla were similar in both cirrhotic and control rats. Receptor autoradiography showed that the upregulation of medullary ET_B in cirrhotic rats was due to an upregulation of ET_B in the inner medullary collecting duct cells.

3 The tissue endothelin-1 concentrations were increased in the renal medulla of cirrhotic rats (Cir: 271 ± 68 pg g⁻¹ wet wt.; Con: 153 ± 36 pg g⁻¹ wet wt., *n* = 8; means ± s.d., *P* < 0.01).

4 The glomerular filtration rate was slightly decreased in cirrhotic rats but not altered after bosentan treatment in either cirrhotic or control rats. Bosentan decreased sodium excretion to a similar extent in both cirrhotic and control rats, whereas water excretion was significantly reduced by both dosages of bosentan in cirrhotic rats only (Cir + vehicle: 12.5 ± 0.62 ml day⁻¹, Cir + 6 mg kg⁻¹ day⁻¹ bosentan: 8.6 ± 1.0 ml day⁻¹; Cir + 100 mg kg⁻¹ day⁻¹ bosentan: 7.4 ± 0.6 ml day⁻¹; *n* = 10; means ± s.e.mean).

5 We therefore suggest that the upregulation of the medullary ET_B in cirrhotic rats is involved in the regulation of water excretion in rats with CCl₄-induced liver cirrhosis.

Keywords: Endothelin; endothelin receptor antagonist; bosentan; liver cirrhosis; paracrine renal endothelin system; kidney dysfunction in liver cirrhosis

Introduction

Endothelins (ET-1, ET-2 and ET-3), a family of 21 amino acid peptides thought to regulate local vasomotor tone, are the most potent vasoconstrictor substances yet identified. Endothelin-1 was the first of the isopeptides to be characterized in the supernatant of cultured vascular endothelial cells (Yanagisawa *et al.*, 1988). The biological effects of endothelin are mediated by plasma-membrane-bound receptors. Three endothelin receptor subtypes (ET_A, ET_B and recently ET_C) have been cloned (Arai *et al.*, 1990; Haendler *et al.*, 1992; Kumar 1994). They belong to the family of rhodopsin-like receptors coupled to a G-protein and differ in their binding affinity to endothelin isopeptides (ET_A: ET-1 ≥ ET-2 > ET-3; ET_B: ET-1 = ET-2 = ET-3, ET_C: ET-3 > ET-2 = ET-1) as well as in their expression by different cell types. Apart from their vasoactive (Lüscher *et al.*, 1993) and renal properties (Gross *et al.*, 1993), endothelins have been shown to induce a wide variety of biological activities in non-vascular tissues (for reviews, see Leppaluoto *et al.*, 1992; Sokolovsky, 1992; Lüscher *et al.*, 1993), including the liver (Gandhi *et al.*, 1992; 1993).

It is well known that plasma endothelin concentrations are significantly increased in rats as well as patients with liver cirrhosis and ascites and that these high ET-1 concentrations

are associated with kidney dysfunction (Claria *et al.*, 1991a; Asbert *et al.*, 1993; Uchihara *et al.*, 1992; Moller *et al.*, 1993; Nanji *et al.*, 1994).

Since endothelin is mainly a paracrine acting hormone and there have been no studies so far concerning the paracrine renal endothelin system in liver cirrhosis, the aim of our study was to analyse the expression of endothelin receptor subtypes and endothelin synthesis in the kidney of rats with CCl₄-induced liver cirrhosis and ascites. Furthermore, we have analysed the function of the paracrine renal endothelin system in cirrhotic rats using the orally active mixed (A/B) endothelin receptor antagonist, bosentan (Clozel *et al.*, 1994).

Methods

Blood-pressure measurement

To measure arterial blood pressure, the rats were placed in a retaining box and warmed until a pulse was detected in the tail. Systolic pressure was measured with a tail cuff and a pressure transducer in conjunction with an automatic pressure delivery system and chart recorder (Harvard indirect rat-tail blood-pressure system, Harvard Apparatus Ltd., Edenbridge, England).

Animals and treatment

All animal experiments were conducted in accordance with local institutional guidelines for the care and use of laboratory

¹ Author for correspondence at: Universitätsklinikum Charité der Humboldt Universität zu Berlin, Abteilung für Nephrologie (Medizinische Klinik 5), Schumannstr. 20–21, 10098 Berlin, Germany.

animals. Male Wistar rats (WKY, Møllegaard, Denmark) were maintained on standard laboratory chow (Altromin, Altromin GmbH, Germany) and tap water *ad libitum* with a 12 h light-dark cycle at constant temperature and humidity. At the time of the experiment, rats weighed about 350 g.

Induction of liver cirrhosis

The liver cirrhosis was induced according to McLean *et al.* (1969). The rats received phenobarbitone water at a concentration of 3 g l^{-1} and were exposed to CCl_4 by inhalation. A box with a transparent cover (150 l capacity) was fitted with an inlet tap. Compressed air was passed into the box via a flow meter (Kendal, Neustadt, Germany), at 21 min^{-1} , bubbling through two wash bottles containing CCl_4 . Air left the box by leaking through the joints. At each dose, the CCl_4 vapour was run in for 5 min and then turned off, and the rats were left in the box for 5 min. This was repeated twice weekly. The rats were treated with CCl_4 inhalation twice a week for 9 weeks. The control rats of the same age and sex were not treated.

Treatment with the mixed (A/B) endothelin receptor antagonist, bosentan

The rats were divided into six age-matched groups. All 6 groups were of 10 rats and treated during 2 days, and bosentan was always freshly prepared and administered as micronized free sulphonamide by gastric gavage. Vehicle was 5% arabic gum. *Group I:* Control rats without bosentan treatment, the rats received vehicle only. *Group II:* Control rats with bosentan treatment ($6 \text{ mg kg}^{-1} \text{ day}^{-1}$). *Group III:* Control rats with bosentan treatment ($100 \text{ mg kg}^{-1} \text{ day}^{-1}$). *Group IV:* Rats with CCl_4 cirrhosis not submitted to bosentan treatment; the rats received vehicle. *Group V:* Rats with CCl_4 cirrhosis with bosentan treatment ($6 \text{ mg kg}^{-1} \text{ day}^{-1}$). *Group VI:* Rats with CCl_4 cirrhosis and with bosentan treatment ($100 \text{ mg kg}^{-1} \text{ day}^{-1}$).

Urine was collected for the determination of pH sodium, potassium and creatinine. All rats were killed after 48 h. Blood samples were collected from the inferior vena cava. The liver was removed quickly. Liver samples for tissue extraction of ET-1 and Scatchard analysis were frozen immediately in liquid nitrogen and stored at -70°C until further use.

Light microscopy

Tissue was obtained from the middle lobe of the liver of each animal. Pathohistological evaluation of the liver morphology (haematoxylin-eosin and elastica van Gieson staining) were performed on all tissue blocks.

Serum enzymes (clinical chemistry) and glomerular filtration rate

Serum levels of potassium, sodium, aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH) and bilirubin were measured with the appropriate kits in an automatic analyzer. Blood samples for the determination of serum enzymes were drawn at the end of the experiment.

Measurement of the glomerular filtration rate The GFR was measured by the inulin single-shot method (Gretz *et al.*, 1990) after 24 h of treatment with bosentan or vehicle (see above). The single-shot clearance was evaluated by displaying a two-compartment model with resolution of the plasma inulin concentrations into two monoexponential functions. The rats received an intravenous i.v. bolus injection of 150 mg of inulin (Inutest). Blood samples for determination of serum inulin concentrations were drawn at 0, 30, 90, 135 and 180 min after injection. Inulin was measured by a modified β -fructosidase method (Kühnle *et al.*, 1992). GFR is expressed as $\text{ml min}^{-1} 100 \text{ g}^{-1}$ body weight.

Endothelin-1 radioimmunoassay

Tissue preparation Liver- and kidney-tissue-immunoreactive endothelin levels were measured by a method described by Bolger *et al.* (1991). After thawing, liver samples were suspended in 2 ml g^{-1} wet wt. of 0.2 M acetic acid, 0.5 M NaCl, disrupted with a polytron and subsequently homogenized. The homogenate was centrifuged at 4°C for 15 min at $25,000 \text{ g}$. The supernatant was stored for endothelin-1 radioimmunoassay and the pellet discarded.

Radioimmunoassay Immunoreactive endothelin-1 (irET-1) was extracted from tissue supernatant using Amprep 500 C2 columns (Amersham). One-ml samples were acidified with 0.25 ml of 2 M HCl, centrifuged at $10,000 \text{ g}$ for 5 min at room temperature and loaded onto the columns. The columns were washed with 5 ml of water and 0.1% trifluoroacetic acid (TFA), and the adsorbed peptide was eluted with 2 ml of 80% methanol in water and 0.1% TFA. The eluents were collected in polypropylene tubes and dried under a stream of nitrogen. The probes were reconstituted in $250 \mu\text{l}$ of assay buffer (0.02 M borate buffer, pH 7.4, containing 0.1% sodium azide), and $2 \times 100 \mu\text{l}$ were taken for analysis in a commercial endothelin-1 ^{125}I radioimmunoassay kit (Endothelin-1,2 (high sensitivity) ^{125}I assay system, Amersham). Separation of the antibody-bound fraction was effected by magnetic separation with the Amerlex-M Separator (Amersham). This assay reacts 100% with ET-1 and cross-reacts 189% with ET-2. Cross-reactivity with ET-3 was less than 0.001 .

ETA/ETB-receptor-binding experiments

Membrane preparation Membranes were prepared according to Nambi *et al.* (1990) and Hoher *et al.* (1995) with minor modifications. Approximately 0.3 g of the kidney was homogenized at 4°C in 20 ml of 20 mmol l^{-1} NaHCO_3 with a motor-driven pestle homogenizer. The homogenate was centrifuged at 4°C for 15 min at 1000 g . The supernatant was decanted and centrifuged at 4°C for 30 min at $40,000 \text{ g}$. The pellet, consisting of crude plasma membranes, was resuspended in assay buffer (100 mM Tris-HCl, 5 mM MgCl_2 , and $0.1 \text{ g}\%$ BSA, pH 7.4) at a protein concentration of $200 \mu\text{g ml}^{-1}$. Protein content was determined according to the method of Lowry *et al.* (1951) with bovine serum albumin used as a standard.

Binding assays In order to analyse the expression of endothelin receptor subtypes (ETA/ETB) in the kidney, binding assays were performed in the presence or absence of the subtype-specific endothelin receptor ligands, BQ123 ($3 \mu\text{M}$) and/or BQ3020 ($3 \mu\text{M}$) (Nambi *et al.*, 1992). The assay buffer for binding studies contained 1 mg ml^{-1} bacitracin, 100 mM Tris-HCl, 5 mM MgCl_2 , and $0.1 \text{ g}\%$ BSA, pH 7.4, in a total volume of $150 \mu\text{l}$. The [^{125}I]-ET-1 tracer concentration was kept constant at $40,000 \text{ c.p.m./tube}$, while the concentration of unlabelled ET-1 was increased from 0 to 25 nM (competition studies with 'cold saturation'). Samples from crude plasma membranes were used at a concentration of $0.53 \text{ mg protein ml}^{-1}$ according to Hoher *et al.* (1992b, 1995). Binding studies were performed at room temperature for 120 min. Non-specific binding was assessed in the presence of excess ET-1 ($5 \mu\text{M}$). After adding 1 ml of cold binding buffer, free and receptor-bound radioactivity was separated by centrifugation at $30,000 \text{ g}$ (4°C) for 15 min, and the pellets thus obtained were washed two additional times with 1 ml of cold binding buffer. [^{125}I] was counted in a Packard Gamma Counter (78% counting efficiency for ^{125}I).

Autoradiography of tissue endothelin receptor subtypes

In vitro receptor autoradiography was performed according to Hoher *et al.* (1992a; 1995) with minor modifications. The

frozen kidneys was cut into 5 μm thick sections with a microtome-cryostat. The sections were placed on precooled slides, air-dried for 10 min and stored at -20°C . Autoradiographic incubation was performed in the presence or absence of subtype specific endothelin receptor ligands BQ123 (3 $\mu\text{mmol l}^{-1}$) and/or BQ3020 (3 $\mu\text{mmol l}^{-1}$). The slides were covered with 100 μl binding buffer (1 g l^{-1} bacitracin, 100 mmol l^{-1} Tris-HCl, 5 mmol l^{-1} MgCl_2 , and 1 g l^{-1} bovine serum albumin, pH 7.4) containing [^{125}I]-ET-1 in a concentration of 50 pM (24000 d.p.m. 100 μl^{-1}) and incubated in a moist chamber for 2 h at room temperature. Radioactivity was withdrawn by suction with a vacuum pump and the slides were washed 4 times for 10 min in 4°C cold binding buffer. Finally the sections were fixed for 20 min in 5% formalin, 2.5% glutaraldehyde, 0.9% NaCl. To remove residual buffer the slides were briefly dipped in distilled water. Autoradiograms with both endothelin receptor ligands (BQ123, BQ3020) served as a control. The sections were layered with photoemulsion (K5 nuclear trace emulsion, Ilford, Germany) and stained with haematoxylin and eosine. Evaluation was done by counting the silver grains.

Chemicals

[^{125}I]-Tyr 13 -endothelin-1 ([^{125}I]-ET-1) (2200 Ci mmol^{-1}) was obtained from Du Pont, Germany. Unlabelled ET-1 was from Peninsula Laboratories, Inc. (Germany). The mixed (A/B) endothelin receptor antagonist, bosentan (4-tert-butyl-N-(2-hydroxy-ethoxy)-5-(2-methoxy-phenoxy)-2,2'-bipyrimidine-4-yl}-benzenesulphonamide) was a generous gift from Dr Martine Clozel (F. Hoffmann-La Roche Ltd, Basel, Switzerland). The selective endothelin receptor ligands (BQ123 and BQ3020) were purchased from California Peptides Inc. (U.S.A.). CCl_4 was obtained from Merck (Darmstadt, Germany). Kits for radioimmunoassays (RIA) of ET-1 were purchased from Amersham (Braunschweig, Germany).

Unless otherwise stated, all reagents were of analytical grade and were purchased from Merck (Darmstadt, Germany), Boehringer-Mannheim (Mannheim, Germany) and Sigma (München, Germany).

Statistical analysis

Statistically significant differences among individual groups were determined by Student's unpaired *t* test.

Results

The liver histology of all rats treated with CCl_4 showed micronodular cirrhosis (Figure 1). All these animals also had

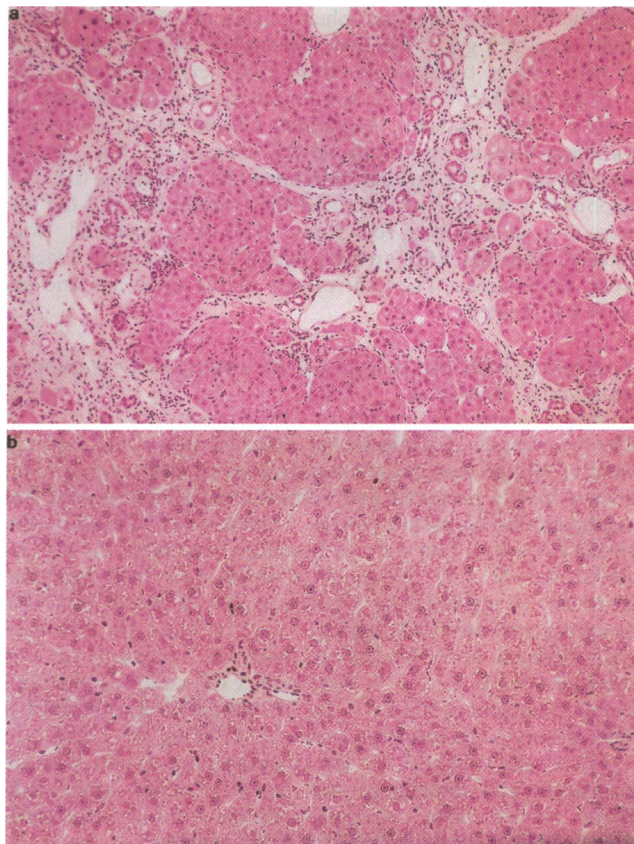


Figure 1 Micronodular liver cirrhosis in the rats treated with phenobarbitone water and CCl_4 inhalation for 9 weeks (a); normal rat liver served as control (b). Calibration bar = (original magnification 166 \times).

Table 1 Serum enzyme activities, sodium-, potassium- and bilirubin-concentrations, blood pressure and glomerular filtration rate in control and cirrhotic rats

	Controls (n = 6)	Liver cirrhosis (n = 6)
Serum alkaline phosphatase (u l^{-1})	169 \pm 42	606 \pm 241 ^{###}
Serum ALT (u l^{-1})	30 \pm 5	74 \pm 29 ^{###}
Serum AST (u l^{-1})	49 \pm 10	160 \pm 63 ^{###}
Serum γ -GT (u l^{-1})	3.17 \pm 0.41	4.83 \pm 1.17 ^{##}
Serum bilirubin (mg l^{-1})	1.8 \pm 0.7	5.0 \pm 2.2 ^{###}
Serum sodium (mmol l^{-1})	138 \pm 1	132 \pm 2 [#]
Serum potassium	4.1 \pm 0.2	3.9 \pm 0.2
MAP (mmHg)	128 \pm 18	110 \pm 18
MAP after 6 mg kg^{-1} day $^{-1}$ bosentan (mmHg)	129 \pm 19	108 \pm 17
MAP after 100 mg kg^{-1} day $^{-1}$ bosentan (mmHg)	122 \pm 21	106 \pm 24
GFR (ml min^{-1} 100 g $^{-1}$ b.w.)	0.85 \pm 0.13	0.67 \pm 0.11
GFR after 6 mg kg^{-1} day $^{-1}$ bosentan (ml min^{-1} 100 g $^{-1}$ b.w.)	0.83 \pm 0.18	0.71 \pm 0.15
GFR after 100 mg kg^{-1} day $^{-1}$ bosentan (ml min^{-1} 100 g $^{-1}$ b.w.)	0.92 \pm 0.16	0.69 \pm 0.12

Serum levels of sodium, potassium and aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase and bilirubin were measured using the appropriate kits in an automatic analyser. MAP: mean arterial blood pressure (mmHg), GFR: glomerular filtration rate. The mixed (A/B) endothelin receptor antagonist bosentan (6 or 100 mg kg^{-1} day $^{-1}$) was given by gastric gavage. Values are means \pm s.d.; [#] $P < 0.05$, ^{##} $P < 0.01$, ^{###} $P < 0.005$, compared to control rats.

ascites at the time of the study, the ascites volume ranging from 2 to 28 ml. Control rats had a normal liver histology and no ascites. Plasma bilirubin was increased in the cirrhotic rats (Table 1). Blood pressure was slightly decreased in cirrhotic rats compared to control rats. The mixed (A/B) endothelin receptor antagonist, bosentan, did not decrease blood pressure in control and cirrhotic rats (Table 1). Scatchard analysis revealed an overexpression of the endothelin B receptor (ET_B) in the renal medulla of rats with cirrhosis (Cir; B_{max} : 2775 ± 299 fmol mg^{-1} ; Con; B_{max} : 1695 ± 255 fmol mg^{-1} ; $n=8$; $P<0.01$), whereas the density of the ET_B in the cortex and of the endothelin A receptor (ET_A) in the cortex and medulla were similar in both cirrhotic and control rats (Table 2). Scatchard analysis revealed only one type of ET_A and ET_B binding site in the renal medulla and cortex of control and cirrhotic rats (Figure 2). The binding constants were equal in all test groups (data not shown). In accordance with the Scatchard data showing an overexpression of the ET_B in the renal medulla, receptor autoradiography (Figure 5) demonstrated an increased density of the ET_B in medullary collecting duct cells of rats with liver cirrhosis (Cir: 19 ± 4 silver grains/cell; Con: 9 ± 3 silver grains/cell; $n=8$; $P<0.01$). The densities of both the ET_A and ET_B were similar in the glomeruli and blood vessels of cirrhotic and control rats (data not shown).

The highest endothelin-1 concentrations were observed in the renal medulla of cirrhotic rats. (Cir: 271 ± 68 pg g^{-1} wet wt., Con: 153 ± 36 pg g^{-1} wet wt. $n=8$; $P<0.01$, Table 2). The plasma endothelin-1 concentrations were also increased significantly ($P<0.05$) in cirrhotic compared to control rats (Table 3). Tissue endothelin concentrations were equal in normal and cirrhotic liver (Table 3).

GFR was slightly decreased in cirrhotic rats but not altered after either the lower or the higher dose of bosentan treatment in cirrhotic or control rats (Table 1). Both dosages of bosentan (6 and 100 mg kg^{-1} day $^{-1}$) decreased sodium excretion to a similar extent in both cirrhotic and control rats. However, only the higher dose of bosentan led to a significant decrease in sodium excretion. The decrease achieved with the lower dosage was not significant (Figure 4). Water excretion, on the other hand, was reduced by 6 and 100 mg kg^{-1} day $^{-1}$ bosentan in cirrhotic rats only (Cir+vehicle: 12.5 ± 2.2 ml d^{-1} , Cir+6 mg kg^{-1} bosentan: 8.6 ± 1.0 ml d^{-1} ; Cir+100 mg kg^{-1} bosentan: 7.4 ± 0.6 ml d^{-1} , Figure 3). Urinary pH was similar in control rats and rats with liver cirrhosis (Cir: pH: 7.0 ± 1.1 ; Con: pH: 7.2 ± 1.2 ; $n=10$) and was not affected by bosentan treatment.

Discussion

Our study revealed an overexpression of the endothelin B receptor (ET_B) in inner medullary tubules of rats with CCl_4 -induced liver cirrhosis, whereas the density of the ET_B in the cortex and of the endothelin A receptor (ET_A) in the cortex

and medulla were similar in both cirrhotic and control rats. The highest endothelin-1 concentrations were also observed in the renal medulla of cirrhotic rats. The GFR was decreased in cirrhotic rats but not altered after bosentan treatment in either

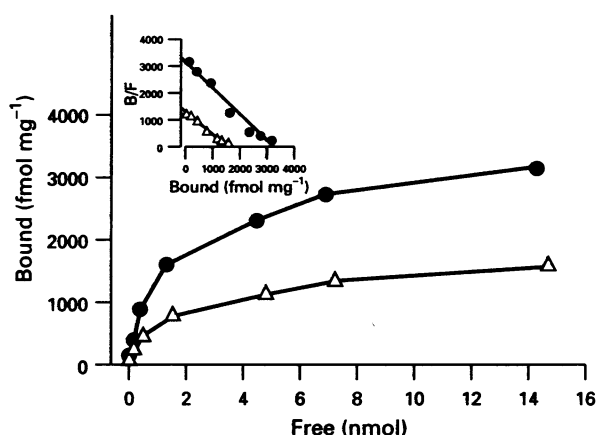


Figure 2 ET_B receptor expression in the renal medulla. The figure shows a representative experiment of [125 I]-ET-1 specific binding after blocking the ET_A with BQ123 to membranes isolated from the renal medulla of a control (Δ) and a cirrhotic (\bullet) rat. Scatchard transformations of binding data (inset) suggest only one type of binding site for the ET_B in both control and cirrhotic rats. Nonspecific binding was determined in the presence of unlabelled ET-1 as described under Methods and was always less than 10% of total binding.

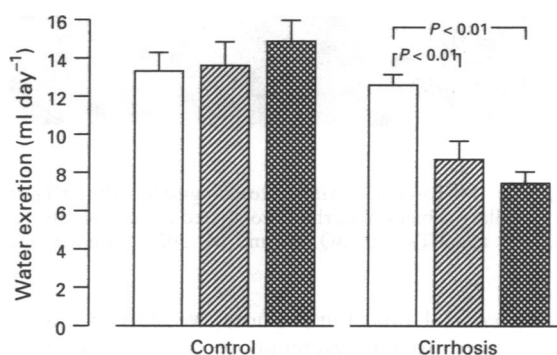


Figure 3 Water excretion was reduced by bosentan in cirrhotic rats only ($n=10$; $P<0.01$). Bosentan had no influence at all on water excretion in control rats. The rats received bosentan 6 (hatched columns) and 100 (cross-hatched columns) mg kg^{-1} day $^{-1}$ respectively or vehicle (open column) by gastric gavage (means \pm s.e.mean).

Table 2 Endothelin receptor density (B_{max}) and tissue endothelin-1 concentration in the renal cortex and medulla in control and cirrhotic rats

	Renal cortex		Renal medulla	
	Control	Liver cirrhosis	Control	Liver cirrhosis
Tissue ET-1	153 ± 37	143 ± 24	153 ± 36	$271 \pm 68^{\#}$
ET_A (fmol mg^{-1})	355 ± 78	312 ± 45	599 ± 59	677 ± 54
ET_B (fmol mg^{-1})	501 ± 76	509 ± 50	1695 ± 255	$2775 \pm 299^{\#}$

The ET_{A1} and ET_B receptor density was derived from Scatchard plots of [125 I]-ET-1 binding studies according to Methods. Nonspecific binding was assessed in the presence of $5 \mu\text{mol l}^{-1}$ of unlabelled ET-1. It ranges between 5–10%. Samples from crude plasma membranes were used at a concentration of $0.53 \text{ mg of protein ml}^{-1}$. Tissue-immunoreactive endothelin-1 (ET-1) was determined with a commercial endothelin-1 radioimmunoassay kit. Values are means \pm s.d. of 8 separate animals in each group. $^{\#}$ indicates $P<0.01$ compared to control rats.

Table 3 Plasma and liver tissue ET-1 concentration in control and cirrhotic rats

	Controls (n = 6)	Liver cirrhosis (n = 6)
Plasma ET-1 (pg ml ⁻¹)	19.8 ± 5.2	30.1 ± 3.9 [#]
Plasma ET-1 after 100 mg kg ⁻¹ day ⁻¹ bosentan treatment (pg ml ⁻¹)	23.7 ± 6.8	33.7 ± 6.2 [#]
Liver tissue ET-1 (pg g ⁻¹)	59 ± 5	53 ± 9

Liver tissue and plasma immunoreactive endothelin-1 (ET-1) was determined using a commercial endothelin-1 radioimmunoassay kit. Bosentan (100 mg kg⁻¹ day⁻¹) was given by gastric gavage. Values are means ± s.d. of 8 separate animals in each group. [#]indicates *P* < 0.05 compared to control rats.

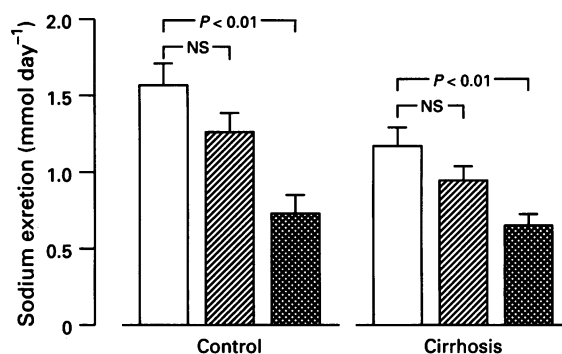


Figure 4 The mixed (A/B) endothelin receptor antagonist bosentan decreased sodium excretion to a similar extent in both cirrhotic and control rats. Bosentan 6 (hatched columns) and 100 (cross-hatched columns) mg kg⁻¹ day⁻¹ respectively or vehicle (open columns) was given by gastric gavage (*n* = 10; means ± s.e.mean).

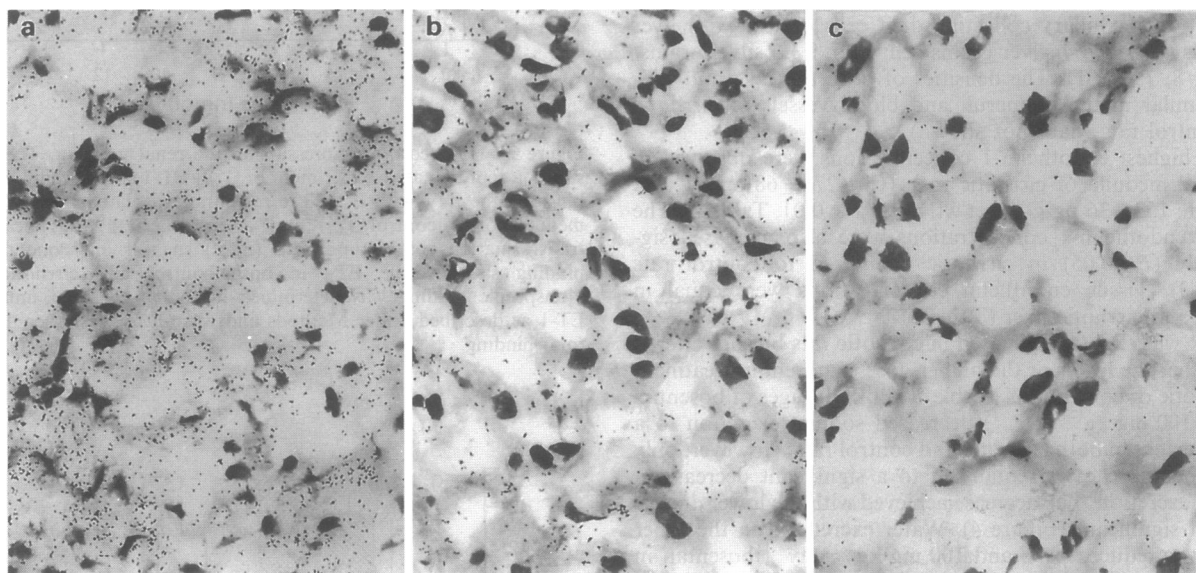


Figure 5 Microscopic *in vitro* autoradiography of the ET_B receptor in rat kidneys showing an upregulation of ET_B receptor protein in medullary tubules of cirrhotic compared to control rats: (a) cirrhotic rat; (b) control rat and (c) nonspecific binding after blocking the ET_A and ET_B with BQ 123 and BQ 3020. (original magnification 830 ×).

cirrhotic or control rats. Furthermore, we showed that bosentan decreased sodium excretion to a similar extent in both cirrhotic and control rats, whereas water excretion was significantly reduced by bosentan in cirrhotic rats only.

Plasma ET-1 concentrations in liver cirrhosis

It is well known that plasma endothelin concentrations are significantly increased in rats as well as in patients with liver cirrhosis and ascites and that the increased plasma endothelin concentrations are associated with kidney dysfunction (Claria *et al.*, 1991a; Uchihara *et al.*, 1992; Asbert *et al.*, 1993; Moller *et al.*, 1993; Nanji *et al.*, 1994). The precise mechanisms of the elevated plasma endothelin concentrations and their pathophysiological role in cirrhosis remain to be clarified. However, several points should be addressed in this context: (i) Circulating endotoxin, as discussed later, is increased both in rats (Nolan, 1975; Yoshimura *et al.*, 1993) and patients with liver cirrhosis and stimulates endothelin synthesis (Vemulapalli *et al.*, 1991). A reduced hepatic ET-1 clearance seems to be unlikely (Asbert *et al.*, 1993). (ii) Increased plasma endothelin concentrations were reported in patients and experimental models with heart failure, hypertension, chronic and acute renal failure without impaired liver function, and cardiogenic shock

(Leppaluoto *et al.*, 1992; Sokolovsky, 1992; Lüscher *et al.*, 1993), indicating that elevated plasma ET-1 concentrations are a rather nonspecific parameter. We suggest that immunoreactive plasma concentrations reflect a spillover from the tissue endothelin system. (iii) We did not see a fall of mean arterial blood pressure after blocking the ET_A and ET_B receptors with bosentan, which indicates that the circulating endothelin as well as the paracrine endothelin system are not involved in the maintenance of arterial blood pressure in cirrhotic rats (Table 1). (iv) Bosentan treatment increased plasma immunoreactive ET-1 in both cirrhotic and control rats (Table 3). Löffler *et al.* (1993) analysed the bosentan analogue Ro 46-2005 and also observed increased plasma immunoreactive ET-1 after Ro 46-2005 treatment. He suggested that Ro 46-2005 induced a displacement of ET-1 from the endothelial ET_B because the elevation of plasma immunoreactive ET-1 occurred very rapidly after treatment with the endothelin receptor antagonist. However, ET-1 gene expression could be increased by blocking the ET_B on endothelial cells with bosentan, because ET_B stimulates nitric oxide formation in endothelial cells. Nitric oxide, via cyclic GMP, is a very potent inhibitor of ET-1 gene expression (Lüscher *et al.*, 1993). Therefore, blocking the ET_B may increase the ET-1 mRNA transcription rate.

Stimulation of ET-1 and ET_B in the renal medulla of rats with CCl₄-induced liver cirrhosis

This study revealed a significantly increased ET_B density and tissue ET-1 concentration in the renal medulla of rats with liver cirrhosis, indicating an upregulation of the paracrine endothelin system in the renal medulla of these animals (Table 2, Figures 2 and 5). This increased synthesis of endothelin in the renal medulla of cirrhotic rats is probably due to the mechanisms discussed below. Endotoxin, a well-known stimulus of ET-1 synthesis (Vemulapalli *et al.*, 1991), is increased in the kidneys of rats with CCl₄-induced liver cirrhosis (Nolan 1975; Yoshimura *et al.*, 1993) as well as in cirrhotic patients (Triger, 1991). The endotoxaemia leading to an increased tissue concentration of endotoxin in rats with liver cirrhosis is thought to result from the combination of an overflow of endotoxin due to decreased detoxifying activity of the liver, a breakdown of the intestinal mucosa barrier due to a diminished secretion of bile acid and changes in bacterial flora, as well as an efflux of portal endotoxin into the systemic circulation due to short-circuits between the portal and systemic circulation (Yoshimura *et al.*, 1993). Second, patients (and rats) with end-stage liver cirrhosis often show a vasoconstriction of the renal arteries (Epstein, 1988) leading to tissue hypoxia. Hypoxia is a well-known stimulus of endothelin-1 synthesis in the kidney (Lüscher *et al.*, 1993; Kohan, 1993b). On the other hand, an increased synthesis of nitric oxide (NO), which has been implicated in the pathogenesis of hypotension in these animals, was demonstrated in rats with liver cirrhosis (Vallance & Moncada, 1991; Clair *et al.*, 1992). Nitric oxide is a very potent endogenous inhibitor of endothelin synthesis (Kourembanas *et al.*, 1993) with one exception: the inner medullary collecting duct cell. The inner medullary collecting duct cell is not submitted to the usual control mechanisms. ET-1 production in these cells is under unique control. NO and its second messenger cyclic GMP only mildly decrease medullary ET-1 release (Kohan & Padilla, 1994) compared to the renal cortex or other tissues. Therefore, endotoxin may increase ET-1 synthesis in the renal medulla, whereas the endotoxin stimulus on ET-1 gene expression in the renal cortex is suppressed by nitric oxide. The mechanisms leading to an ET_B overexpression in the renal medulla of cirrhotic rats are as yet unknown. However, several points should be addressed in this context: (i) Upregulation of a hormone usually causes downregulation of the corresponding hormone receptor and *vice versa*. This was also demonstrated for the paracrine endothelin system (Clozel *et al.*, 1993; Roubert *et al.*, 1993b). On the other hand, our study showed that there was no such a negative feedback in the renal medulla of rats with liver cirrhosis. We found an upregulation of both the ET-1 tissue concentration and the ET_B density in the renal medullary cirrhotic rats. (ii) Well-known stimulants of ET_B upregulation in rat cultured vascular smooth muscle cells are natriuretic peptides such as the C type natriuretic peptide and atrial natriuretic peptide (Eguchi *et al.*, 1994). Provided that natriuretic peptides also increase the expression of ET_B in renal inner medullary collecting duct cells, the increased plasma levels of natriuretic peptides in rats with liver cirrhosis (Gines *et al.*, 1988; Laffi *et al.*, 1989) may contribute to the ET_B overexpression. (iii) An upregulation of ET_B in the renal medulla was observed in rats with acute renal failure after administration of hypertonic glycerol leading to tissue hypoxia (Roubert *et al.*, 1993a). An impaired renal microcirculation with tissue hypoxia is most probably the cause of ET_B upregulation in this case. An impaired renal microcirculation is also observed in rats with liver cirrhosis. Therefore, we suggest that tissue hypoxia in the kidneys of cirrhotic rats may also contribute to the ET_B overexpression in the renal medulla. Roubert *et al.* (1993a) proposed that the pronounced ET_B overexpression in the renal medulla of rats with acute renal failure may account for or contribute to functional renal impairment. However, they did not prove this theory using endothelin receptor antagonists.

Effect of bosentan on water and sodium excretion

Bosentan was chosen to analyse the pathophysiological relevance of the alterations in the paracrine renal endothelin system in cirrhotic rats (increased ET_B density within the medullary tubules and increased ET-1 tissue concentration in the renal medulla), because it was shown that bosentan did not influence the renal blood flow, the renal vascular resistance or the glomerular filtration rate in conscious chronically instrumented rats (Hoher *et al.*, 1995) and dogs (Teerlink *et al.*, 1995) thus allowing an independent analysis of tubular functions, whereas ET-1 and ET_B agonists like sarafotoxin 6c (Warner *et al.*, 1993) markedly decreased renal blood flow thus not allowing an independent analysis of tubular functions. Bosentan differs in its *in vivo* potency in blocking ET_A- and ET_B-mediated effects. Blockade of ET-1-induced ET_A-mediated effects requires higher dosages of bosentan compared to the blockade of the ET-1-induced ET_B-mediated effects (Clozel *et al.*, 1994; Hoher *et al.*, 1995). The reasons for these observations are not yet clear. Clozel *et al.* (1994) suggested that bosentan reaches effective concentrations in the (ET_B-rich) endothelial layer more easily than in the (ET_A-rich) media of blood vessels. In any case, use of lower and higher dosages of bosentan makes it possible to distinguish between ET_A- and ET_B-mediated effects *in vivo*. In a recent study, we demonstrated that as little as 3–10 mg kg⁻¹ of bosentan, i.v., resulted in a markedly decreased (ET_B-mediated) depressor response to ET-1, whereas the pressor response (ET_A-mediated) was not altered. Use of 100 mg kg⁻¹ of bosentan i.v., on the other hand, blocked both the ET_A- and ET_B-mediated responses to ET-1 (Hoher *et al.*, 1995). Based on these pharmacological characteristics of bosentan, we demonstrated in our study using both dosages of bosentan that the low bosentan dosage reduced water excretion in cirrhotic rats to an extent similar to that achieved with the higher one, suggesting that the bosentan effect on water excretion is ET_B-mediated. The additional blockade of ET_A by the higher dose of bosentan obviously has no additive effect.

The decreased water excretion in cirrhotic rats after bosentan treatment may be due to the well-known endothelin-1-induced, ET_B-mediated inhibition of vasopressin-induced water reabsorption (Edwards *et al.*, 1993). Via ET_B receptors endothelin-1 inhibits vasopressin-dependent cyclic adenosine 3':5'-monophosphate accumulation and vasopressin-stimulated water permeability in the collecting duct, thus decreased urine osmolarity and increasing urine flow (Kohan, 1993a). Cirrhotic rats had elevated plasma vasopressin levels with a resultant vasopressin-dependent decrease in water excretion (Arroyo *et al.*, 1988; Clair *et al.*, 1991b) and also exhibited an increased ET_B density in the renal medulla, as shown in our study by two independent methods. Using bosentan, we blocked the ET_B-mediated inhibitory effect of endothelin-1 on vasopressin-induced water reabsorption. Such a blockade of ET_B would result in less autocrine inhibition of vasopressin-dependent water transport with a subsequent decrease of urine flow. Therefore, the upregulation of medullary ET_B in rats with liver cirrhosis may be a protective mechanism against water retention in these animals.

Since we found no differences of glomerular ET_A and ET_B expression between cirrhotic and normal rats, it was not surprising that the effects of blocking glomerular ET_A and ET_B with bosentan in cirrhotic and normal rats were similar with respect to glomerular filtration rate. The lower dose of bosentan (mainly ET_B blockade, see above) decreased sodium excretion non-significantly, whereas the higher one (ET_A and ET_B blockade) resulted in a significant reduction of sodium excretion in both cirrhotic and control rats, indicating that sodium excretion seems to be ET_A-mediated. An ET_A-dependence of tubular sodium excretion was also reported by Gellai *et al.* (1994). Na⁺-K⁺-ATPase and Na⁺/PO₄-exchanger seem to play a major role in these ET_A-mediated effects of endothelin-1 (Garvin & Sanders, 1991).

In conclusion, this study demonstrated that both the tissue endothelin concentration and ET_B density are significantly

increased in the renal medulla of rats with CCl₄-induced liver cirrhosis compared to control rats. Blockade of this activated endothelin system with the orally active endothelin receptor antagonist, bosentan, resulted in a decreased water excretion in cirrhotic rats, indicating that the observed alterations in the renal paracrine endothelin system contribute to the regulation of water excretion in rats with liver cirrhosis and ascites.

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