LIVER AND BILIARY DISEASE

Endothelin is an important determinant of renal function in a rat model of acute liver and renal failure

R Anand, D Harry, S Holt, P Milner, M Dashwood, D Goodier, M Jarmulowicz, K Moore

Gut 2002;**50**:111–117

See end of article for authors' affiliations

Correspondence to: Dr K Moore, Centre for Hepatology, Department of Medicine, Royal Free and University College Medical School, Royal Free Campus, Rowland Hill St, London NW3 2PF, UK; kmoore@rfc.ucl.ac.uk

Accepted for publication 29 March 2001

Background and aims: Renal failure occurs in approximately 55% of patients with acute liver failure. We have previously shown that plasma endothelin 1 concentrations are elevated in patients with acute liver failure and the hepatorenal syndrome. There are few reported satisfactory animal models of liver failure together with functional renal failure. In this study, a rat model of acute liver failure induced by galactosamine that also develops renal failure was first characterised. This model was used to investigate the hypothesis that endothelin 1 is an important mediator involved in the pathogenesis of renal impairment that occurs in acute liver failure.

Methods: Acute liver failure was induced in male Sprague-Dawley rats by intraperitoneal injection of galactosamine together with treatment with the endothelin receptor antagonist Bosentan. Twenty four hour urine collections were made using a metabolic cage. Renal blood flow was measured in anaesthetised animals.

Results: This model developed renal failure and liver failure in the absence of any significant renal pathology, and with an accompanying fall in renal blood flow. Plasma concentrations of endothelin 1 were increased twofold following the onset of liver and renal failure ($p<0.05$), and there was significant upregulation of the endothelin receptor A (ET_A) in the renal cortex ($p<0.05$). Administration of Bosentan prevented the development of renal failure when given before or 24 hours after the onset of liver injury ($p<0.05$) but had no effect on liver injury itself, or on renal blood flow.

Conclusions: This study demonstrates that this animal model has many of the features needed to be regarded as a model of renal failure that occurs in acute liver failure. The observation that plasma levels of endothelin 1 and ET_A receptors are increased and upregulated, and that renal failure is prevented by an endothelin antagonist supports the hypothesis originally put forward that ET_A is important in the pathogenesis of renal failure that occurs in patients with acute liver failure.

Renal failure occurs in 40–80% of patients with end stage liver disease and is associated with an unfavourable prognosis.[1] The development of renal failure in the absence of clinical, anatomical, or pathological causes of renal failure is termed the hepatorenal syndrome (HRS).[2] Classically, HRS is associated with end stage cirrhosis. However, it is widely recognised that renal failure occurs spontaneously in approximately 50% of patients with acute liver failure,[3 4] and recognition that HRS may develop in acute liver failure is now specifically included within the most recent definition of HRS.[5]

One of the hallmarks of HRS is that there are relatively few histological changes in the kidneys, and that renal failure is secondary to haemodynamic and functional changes in the kidney. It has been known for many years that patients with severe liver failure have a reduction in renal blood flow, indicative of renal vasoconstriction, and that this is most marked in those patients who develop HRS.[6 7] However, studies have demonstrated that other factors must be involved as the decrease in glomerular filtration rate (GFR) is not always proportional to renal blood flow.[8] This suggests that events independent of renal vasoconstriction may be involved in the pathogenesis of this syndrome involving a decrease in the filtration fraction and/or decrease in the glomerular capillary ultrafiltration coefficient (K_f). For this reason, several studies have focused on mediators that can cause both renal vasoconstriction and contraction of mesangial cells, and thus a reduction in both renal blood flow and K_f.[9]

Endothelin 1 (ET-1) was initially identified by Yanagisawa *et al* and is one of the most potent vasoconstrictors in biological systems.[10] Infusion of ET-1 causes renal vasoconstriction and a reduction in GFR.[11–13] Subsequent studies in human volunteers demonstrated that increasing the plasma concentration of ET-1 from basal levels to approximately 10 pmol/l caused a significant reduction in renal blood flow, GFR, sodium excretion, and urinary flow rate,[14] demonstrating that circulating ET-1 can affect renal function. Various cells within the kidney, including the vascular endothelial, tubular epithelial, and mesangial cells either express mRNA for ET-1 or can synthesise ET-1,[15 16] and ET-1 may therefore have a paracrine or autocrine effect. There are two classes of ET-1 receptors—ET_A and ET_B. In the renal cortex of the rat there is an equal distribution of both ET_A and ET_B receptors whereas ET_B receptors predominate (70%) in the renal medulla.[17 18]

Several studies have shown that plasma ET-1 concentrations are elevated in patients with severe liver disease,[19–21] and these levels are highest in patients with acute liver failure and HRS.[19] Ten of 11 patients with HRS had underlying alcoholic hepatitis or acute non-A non-B hepatitis. In this study there was a ninefold elevation of plasma ET-1 concentrations in those developing HRS. However, demonstration of increased ET-1 concentrations in patients with HRS does not necessarily confer causality.

Many studies have investigated the mechanism of galactosamine (GalN) induced hepatoxicity, which in rats causes a syndrome similar to acute hepatic failure.[22] Following a single injection of high dose GalN, rats develop acute liver failure

Abbreviations: HRS, hepatorenal syndrome; GFR, glomerular filtration rate; K_f, ultrafiltration coefficient; ET-1, endothelin 1; ET_A, ET_B, endothelin receptors A and B, respectively; GalN, galactosamine; ALT, alanine transaminase; LDH, lactate dehydrogenase.

with development of a hyperdynamic circulation.[23] Recently, it was reported that GalN induced liver injury is associated with the development of renal failure.[24] However, no data on either the mechanism of renal failure or histological data on whether renal injury occurs were given.

In this study, we have characterised the functional renal changes that occur following the development of acute liver failure induced by GalN in the rat. Using this model, we have investigated the hypothesis that ET-1 is an important modulator of renal function in liver disease.

MATERIALS AND METHODS

Reagents

Unless otherwise stated, all reagents were purchased from Sigma (Poole, Dorset UK).

Animals

All animal experiments were conducted according to Home Office guidelines under the Animals in Scientific Procedures Act 1986. Male Sprague-Dawley rats (body weight 250–300 g) were obtained from the Comparative Biology Unit (CBU), Royal Free and University College School of Medicine. All animals were housed in the CBU and maintained on a standard diet, with a light:dark cycle of 12 hours, at a temperature of 19–23°C, and humidity of approximately 50%.

Rats were placed individually in polycarbonate metabolic cages with free access to food and water. A 24 hour urine sample was collected immediately prior to GalN or saline injection and at 24–48 hours after injection. Animals were sacrificed by exsanguination under anaesthesia (60 mg/kg Inactin; 5-ethyl 5-[1-methylpropyl]-2-thiobarbituric acid) 48 hours post GalN/saline injection. Blood samples were either collected into ethylenediamine tetraacetic acid containing tubes or allowed to coagulate in plain tubes, centrifuged at 800 *g* for 10 minutes, and the plasma stored at −80°C until analysis. Liver and kidney tissue were collected for histology, autoradiography, or regional synthesis of ET-1.

Treatments

D(+)GalN hydrochloride (GalN, molecular weight 216) was administered as an intraperitoneal injection of 1.1 g/kg (5 mmol/kg) as a 200 mg/ml solution in saline (pH 6.8). Controls received 1 ml of saline intraperitoneally. Bosentan (Ro 47-0203/029), a combined ET_A and ET_B receptor antagonist[25] (a gift from Martine Clozel, Actelion Ltd, Allschwil, Switzerland), was administered daily by gavage (orally) at 100 mg/kg in a 5% gum arabic suspension. Sham controls for this treatment received 1 ml of 5% gum arabic solution alone.

The animals were divided into five groups by treatment: group 1 (control group, n=12) was given 1 ml of saline intraperitoneally; group 2 (GalN group, n=12) was administered 1.1 g/kg GalN intraperitoneally; group 3 (Bosentan controls, n=4) received Bosentan orally and saline intraperitoneally; group 4 (Bosentan pre-GalN, n=8) received Bosentan orally 24 hours prior to GalN intraperitoneally; and group 5 (Bosentan post-GalN, n=12) received Bosentan 24 hours after GalN.

Biochemical studies

Blood was collected from the inferior vena cava into ethylenediamine tetraacetic acid or plain tubes and centrifuged at 2000 *g* for 10 minutes at 4°C and plasma or serum stored at −80°C. Serum and urine creatinine were measured by a kinetic colorimetric method (Hitachi Auto-analyser, Japan). This method involves converting bilirubin to biliverdin with potassium hexacyanoferrate (III), and uses a dual wavelength absorption method, eliminating interference from bilirubin. Creatinine clearance was calculated from urine and serum creatinine values. Liver function tests, including alanine transaminase (ALT) and aspartate transaminase, albumin and bilirubin, as well as plasma and urine sodium were determined by the auto-analyser (Hitachi, UK). Urine osmolality was measured as depression of the freezing point (Advanced Micro Osmometer 3300, Adv. Inst. Inc, Massachusetts, USA), and plasma osmolality was calculated from serum sodium, potassium, and urea concentration. Osmolar and free water clearances were calculated as shown below:

$$\text{osmolar clearance } (C_{osm}) = U_{osm} \times V / P_{osm}$$

and

$$\text{free water clearance } (C_{H2O}) = V - C_{osm}$$

where V is 24 hour urine volume, and results are expressed as ml/day.

Histology

For light microscopy, liver and kidney were fixed in formalin and embedded in paraffin blocks. Sections (3 μm) were cut and stained with haematoxylin and eosin (n=6 in both control and GalN rats). For electron microscopy studies, kidneys were fixed in a glutaraldehyde fixative solution (1% paraformaldehyde, 1.5% glutaraldehyde in phosphate buffered saline) for 48 hours at 4°C. Sections (90 nm) were cut, stained with uracyl acetate and Reynold's lead citrate (80 mM $Pb(NO_3)_2$, 120 mM trisodium citrate, 160 mM NaOH), and examined with a Philips 201 transmission electron microscope up to a maximum magnification of 7500 (n=6 in both control and GalN rats).

Determination of toxicity of galactosamine on tubular cells

To determine whether GalN had any direct nephrotoxic effect, cultured renal tubular cells were incubated with GalN, and cell injury quantified by measuring release of lactate dehydrogenase (LDH). LLCPK1 cells (European Collection of Animal Cell Cultures) were cultured in Dulbecco's modified Eagle's medium (Sigma, Poole, Dorset, UK) supplemented with 10% heat inactivated fetal calf serum, streptomycin (70 μmol/l), and penicillin (70 μmol/l). GalN dissolved in saline was added at a final concentration of 10 μM to 50 mM to the cells. Following incubation for 24 hours, the medium was collected. To determine the maximal possible release of LDH, a total cell lysate was prepared by collecting cells in their medium, sonicating, and centrifuging at 2000 *g* for 10 minutes. The supernatants and total cell lysate were tested for LDH activity (Sigma). LDH of the total cell lysate was used for calculating the maximal percentage leakage of LDH. Cell viability after 24 hours of incubation with GalN was assessed by trypan blue exclusion. Experiments were performed in triplicate on three separate cell preparations.

Endothelin 1 measurement in plasma

Endothelin was extracted from plasma of control rats (n=7) and GalN rats (n=8) using Amersham's Amprep 500 mg C2 columns and ET-1 was quantified using the Biotrak ET-1 inhibition enzyme linked immunosorbent assay system (Amersham Pharmacia Biotech Ltd, UK). This assay has an intra-assay coefficient of variation of 6%.

Endothelin 1 measurement in kidney

For measurements of ET-1 in the kidney and for in vitro autoradiography studies, a separate group of rats was used. They were treated exactly as before—that is, controls were injected with saline and the experimental group were injected with 1.1 g/kg GalN. The animals were sacrificed after 48 hours. To measure regional synthesis of ET-1, fresh kidneys were dissected into the cortex, inner medulla, outer cortex, and pelvis from control rats (n=6) and GalN rats (n=6). Sections were immediately frozen in liquid nitrogen until ready for

Table 1 Serum biochemistry at 48 hours after injection of galactosamine (GalN)

Treatment	Bilirubin (μmol/l)	ALT (IU/l)	Albumin (g/l)
Control	0.8 (0.1)	63 (4)	31 (1.0)
GalN	148 (6)***	8857 (1057)***	28 (0.7)*
Bos control	0.5 (0)	77 (11)	32 (0.5)
Bos pre-GalN	118 (20)***	8556 (1754)***	27 (0.5)*
Bos post-GalN	108 (5)***	9191 (933)***	27 (0.6)*

Rats injected with GalN (n=12) developed severe liver injury with significantly elevated levels of bilirubin, alanine transaminase (ALT), and albumin compared with controls (n=12). Rats treated with Bosentan either 24 hours prior to or 24 hours post GalN injection had a similar degree of liver injury.
Groups: control (n=12), GalN (GalN) (n=12), Bosentan+saline (Bos control) (n=4), Bosentan given 24 hours prior to GalN injection (Bos pre-GalN) (n=8), and Bosentan given 24 hours after GalN injection (Bos post-GalN) (n=12).
Values are mean (SEM).
*p<0.05, ***p<0.001, compared with control animals.

analysis. Weighed samples were boiled in 0.5 M acetic acid for 15 minutes, homogenised, and centrifuged for 15 minutes at 3500 *g*. The supernatants were collected and ET-1 levels quantified using an inhouse inhibition enzyme linked immunosorbent assay, as described in detail by Bodin and colleagues.[26]

In vitro autoradiography of kidney sections

For these studies, kidneys from five control animals and five GalN rats were quickly removed and sliced in half. Each kidney block included cortex and medulla. The in vitro autoradiography technique used to determine the ET binding sites was described by Dashwood and colleagues.[27] ET_A binding sites were identified by incubating the sections with the radioligand [^{125}I]-PD151242[28] and ET_B sites with [^{125}I]-BQ3020.[29]

Effect of Bosentan on mean arterial pressure and renal blood flow

Rats for haemodynamic studies were anaesthetised with a single intraperitoneal injection of 0.6 mg/kg Inactin and maintained at 36–37°C on a heating pad with oxygen supplied by face mask. The femoral artery and contralateral vein were exposed and cannulated with 22 gauge Abbocath cannulae (Abbott, Republic of Ireland). Saline was infused into the vein at a rate of 15 ml/h to replace fluid losses. Haematocrit measurements were taken every 15 minutes over a period of one hour and showed no significant differences. Arterial blood pressure readings were recorded using a pressure transducer connected online to the artery and to a MacLab system (ADI instruments). A ventral midline incision was made and the intestines were displaced out of the abdominal cavity and wrapped in a moist swab and then in Clingfilm to decrease heat and fluid loss. An ultrasound Doppler flow probe (Transonic animal research flowmeters, T106/T206 series; Transonic Systems Inc, New York, USA)[30] was hooked under the renal artery of the left kidney and connected to the flowmeter. This measures renal blood flow to within ±3% accuracy. Renal arterial pressure and renal blood flow rate were monitored for 30 seconds every three minutes for the duration of the experiment. Mean arterial pressure and renal blood flow were then calculated and averaged over each 30 second period. To examine the effect of Bosentan on renal haemodynamics in rats with acute liver failure, Ro 47-0203/001, which is the water soluble sodium salt of Bosentan that is suitable for intravenous use, was infused as a dose of 30 mg/kg over 15 minutes, and renal blood flow was measured.

Statistical analysis

All results are expressed as mean (SEM). Statistical analysis was performed using ANOVA and the limit of significance was taken as $p<0.05$. Where appropriate, the Mann Whitney U test was used to analyse non-parametric data when multiple comparisons were not required.

RESULTS

Effect of galactosamine on liver and renal function

Effect of galactosamine on liver function

Rats given 1.1 g/kg GalN by intraperitoneal injection developed liver injury by 48 hours. This was confirmed by an increase in serum bilirubin from 0.8 (0.1) to 148 (6) μmol/l ($p<0.001$) and in ALT from 63 (4) to 8857 (1057) IU/l ($p<0.001$) in control and GalN rats, respectively (table 1). Serum albumin decreased significantly from 31 (1.0) to 28.4 (0.7) g/l ($p<0.05$). Histological examination of haematoxylin and eosin stained sections of liver confirmed the development

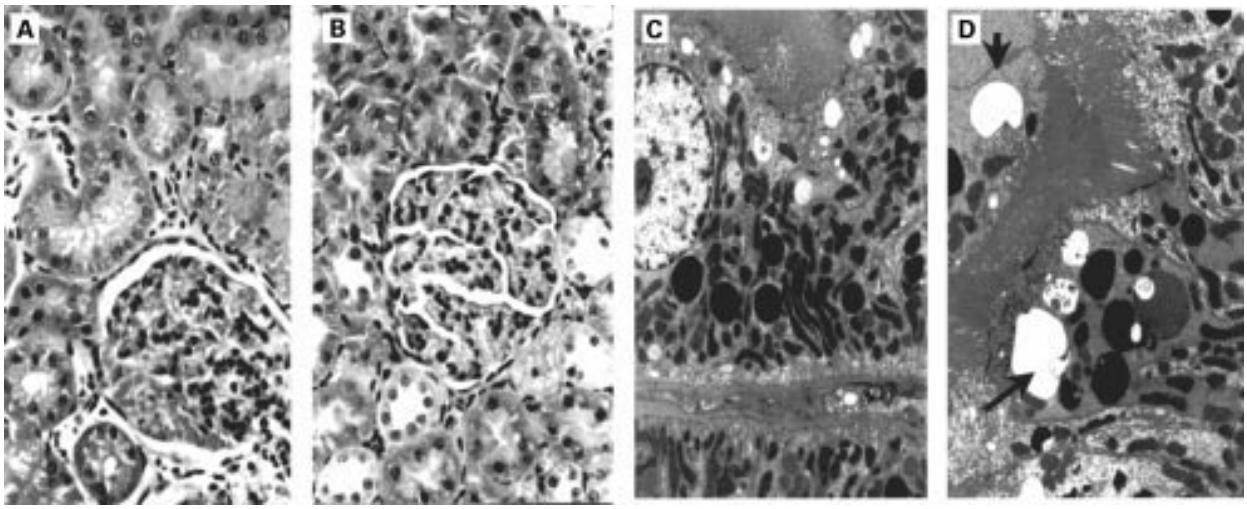

Figure 1 Morphological changes in the kidney. Light micrograph (original magnification ×25) of kidney from (A) sham rats and (B) 1.1 g/kg galactosamine (GalN) injected rats at 48 hours. Kidney sections were fixed in formalin, paraffin embedded, and stained with haematoxylin and eosin. The kidneys showed no abnormality of the renal cortex or medulla in the GalN animals; n=6 in both groups. Electron micrograph (original magnification ×7500) of (C) kidney from sham rats and (D) kidney from 1.1 g/kg GalN injected rats at 48 hours. Kidney sections were fixed in glutaraldehyde and stained with uracyl acetate and Reynold's lead citrate. In GalN animals, the glomeruli were normal. However, in the proximal tubules, the vacuolar system was slightly more prominent, with larger apical vacuoles as well as more prominent vacuoles with flocculent proteinaceous material towards the base of the epithelial cells; n=6 in both groups.

Table 2 Regional distribution of endothelin 1 (ET-1) levels in the kidney of control rats and rats with galactosamine (GalN) induced liver/renal failure

Kidney region	Control (n=6) (pmol/g)	GalN (n=6) (pmol/g)
Cortex	517 (66)	508 (54)
Outer medulla	1697 (286)	1612 (134)
Inner medulla	349 (82)	396 (55)
Pelvis	845 (144)	1085 (125)

Values are mean (SEM) values/g of wet tissue.
There were no significant differences between the two groups although there were clearly regional differences in the formation of ET-1.

of massive hepatocellular necrosis in those animals given GalN (data not shown). Treatment of GalN rats with Bosentan, either before (group 4) or after (group 5) GalN, had no effect on the severity of liver injury at 48 hours (table 1).

Effect of galactosamine on renal function

Control and GalN rats had similar baseline renal function. Following GalN, a 50% decrease in creatinine clearance (1.03 (0.06) to 0.54 (0.03) ml/min; $p<0.001$), together with an increase in serum creatinine (38 (1) to 66 (2) μM; $p<0.01$), and serum urea (5.3 (0.2) to 9.8 (0.8) mmol/l; $p< 0.01$) were observed. Urinary sodium excretion decreased from 1.24 (0.18) to 0.80 (0.08) mmol/day ($p<0.05$) compared with controls which remained unchanged (from 1.20 (0.05) to 1.20 (0.07) mmol/day). Fractional sodium excretion however increased slightly from 0.56 (0.03)% in control rats to 0.78 (0.07)% in those given GalN ($p<0.05$), and was accompanied by a significant increase in urine volume (11.7 (2.2) *v* 16.3 (2.5) ml/day) ($p<0.05$, Wilcoxon matched pairs test). To assess the effects of GalN induced liver failure on the ability of the kidneys to concentrate urine, a cohort of eight animals was studied before and after the onset of liver failure, and data analysed using a paired *t* test. There was no significant difference in urine osmolality before (1477 (258) mosmol/kg water) or after (1009 (116) mosmol/kg water) the onset of acute liver failure (NS). Indeed, the kidneys retained the ability to concentrate urine in rats with GalN induced liver failure, and the urine:plasma osmolality ratio was 3.1 (0.4) in rats with acute liver failure and 4.9 (0.9) prior to injection of GalN, equating to an osmolar clearance of 36.8 (2.6) and 27.6 (3.9) ml/day, respectively (NS). Moreover, free water clearance was virtually identical before (–20.3 (4) ml/day) and after (–22.0 (1.9) ml/day) the onset of acute liver failure (NS). These data confirm that renal tubular function is intact in animals that develop acute liver and renal failure.

Renal histology and electron microscopy studies

Histological examination of the kidneys following haematoxylin and eosin staining from six of the GalN injected rats showed no abnormalities (fig 1B). Examination of these kidneys by electron microscopy demonstrated normal glomeruli (fig 1D). However, the vacuolar system was slightly more prominent in the proximal tubules, with larger apical vacuoles and prominent vacuoles with flocculent proteinaceous material towards the base of the epithelial cells. This system of vacuoles forming part of the endosome/primary and secondary lysozyme system is thought to perform the complex task of endocytosis of macromolecules, including proteins from the filtrate. The significance of this increased prominence of the vacuolar system in GalN rats is unknown.

LDH leakage of LLCPK$_1$ cells

GalN had no effect on release of LDH from cultured LLCPK$_1$ cells. Leakage of LDH in controls was approximately 9.5%, and cells treated with 0.01–50 mM GalN showed leakage of LDH

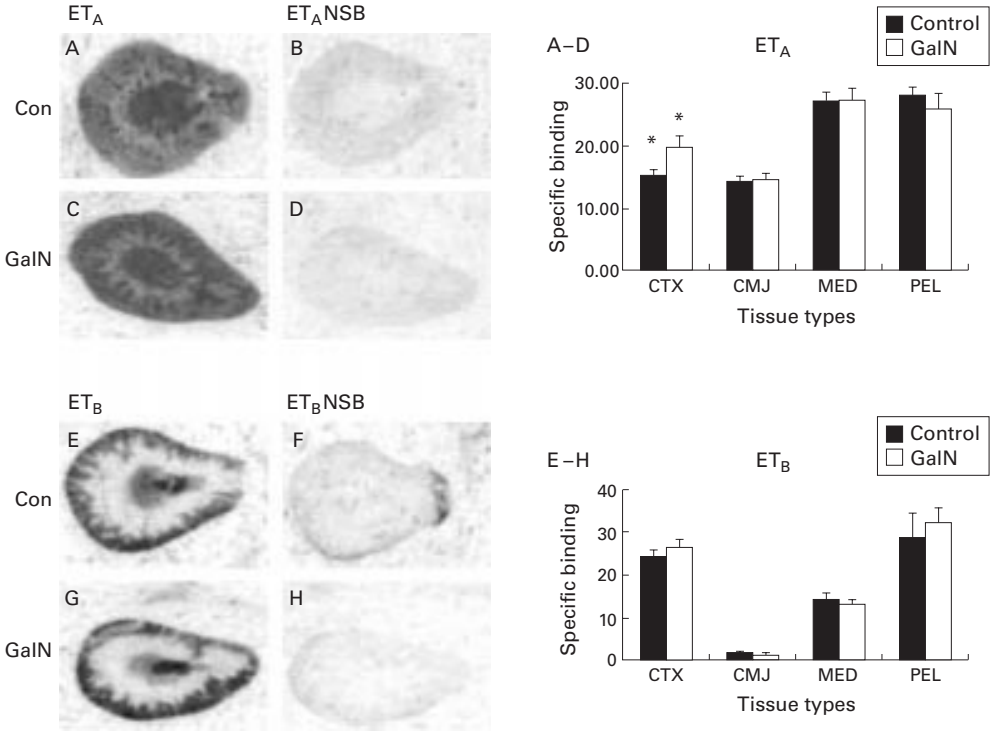

Figure 2 Effect of acute liver failure on endothelin 1 (ET-1) receptor expression in the kidney. Specific binding (total–non specific binding) of endothelin receptor A and B (ET_A and ET_B) selective radioligands [^{125}I]-PD151242 (A–D) and [^{125}I]-BQ3020 (E–H), respectively, in the different sections of the kidney in saline (Con) and galactosamine (GalN) injected rats. Densitometric analysis of ET_A and ET_B receptor binding demonstrated that there was a 20% increase in expression of ET_A receptor in the renal cortex of those animals injected with GalN ($p<0.05$); n=5 in both groups. Values are mean (SEM). CXT, cortex; CMJ, corticomedullary junction; MED, medulla; PEL, pelvis. Autoradiographs depicting total and non-specific binding of [^{125}I]-PD151242 and [^{125}I]-BQ3020 in control and GalN rats.

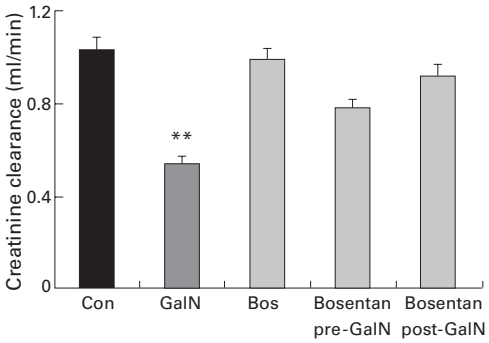

Figure 3 Effect of Bosentan on creatinine clearance. Creatinine clearance decreased significantly in rats with acute liver failure (galactosamine (GalN)) compared with controls (Con) (**p<0.001). Treatment with Bosentan, either before (pre-GalN) of after (post-Galn), prevented the development of renal failure (p<0.05). Groups: Con (n=12), GalN (n=12), saline+Bosentan (Bos) (n=4), Bosentan given 24 hours prior to GalN injection (pre-GalN) (n=8), or Bosentan given 24 hours after GalN injection (n=12) (post-GalN). Values are mean (SEM).

ranging from 9.0% to 10.6%. After administration of GalN to cells, they were tested for viability by exclusion of trypan blue. This revealed <5% staining and viability of the cultured cells was in excess of 95%.

Endothelin 1 as a mediator of renal dysfunction in acute liver failure

Plasma endothelin 1 concentrations

Plasma ET-1 concentrations were increased in rats with GalN induced liver and renal failure (n=8) compared with control animals (n=7) at 44 (8) and 20 (4) pmol/l, respectively (p<0.05).

Renal synthesis of endothelin 1

To determine whether there was increased synthesis of ET-1 by the kidneys, tissue concentrations of ET-1 were determined in the renal cortex, outer medulla, inner medulla, and pelvis. ET-1 concentrations were highest in the renal medulla. There was however no significant difference between ET-1 levels present in the different regions of the kidney in control or GalN treated rats (table 2).

Expression of ET_A and ET_B receptors in the kidney

Expression of ET_A and ET_B receptors in the kidney was determined by autoradiography (fig 2). On initial inspection, it was noted that there was a tendency for greater binding of ET_A receptor ligand in the cortex (see fig 2C), and this was confirmed by densitometric analysis. Quantification of ET_A and ET_B receptor binding demonstrated that there was a 20% increase in expression of ET_A receptors in the renal cortex of those animals injected with GalN (p<0.05) (fig 2). However, there were no further differences observed in either ET_A or ET_B receptor binding in the other regions of the kidney.

Effect of Bosentan on renal function

Administration of Bosentan prevented the development of renal failure in this model. Creatinine clearance decreased from 1.03 (0.06) to 0.54 (0.03) ml/min in those animals given GalN. This was partially prevented by pretreatment with Bosentan, which increased creatinine clearance from 0.54 (0.03) to 0.78 (0.04) ml/min (p<0.001), and GFR was also increased in those animals given Bosentan 24 hours after GalN (0.54 (0.03) to 0.92 (0.05) ml/min; p<0.001) (fig 3). Treatment post GalN was significantly better than treatment pre GalN (p<0.05).

Table 3 Haemodynamic studies in control and galactosamine (GalN) rats treated with Bosentan

Treatment	MAP (mm Hg)	RBF (ml/min/kidney)	RVR (mm Hg/ml/min)
Control (n=12)	115 (3.8)	2.9 (0.2)	44 (4)
GalN (n=12)	106 (5)	1.8 (0.2)***	69 (9)***
Control+Bos (n=4)	113 (5)	2.7 (0.5)	46 (9)
GalN+Bos (n=6)	118 (7)	2.0 (0.4)***	72 (15)***

Values are mean (SEM).
There were no significance differences in mean arterial pressure (MAP). Renal blood flow (RBF) and renal vascular resistance (RVR) were decreased and increased, respectively, in rats with GalN induced liver/renal failure (***p<0.0001). Bosentan had no effect on renal blood flow or renal vascular resistance in either control animals or those with liver failure.

Effect of Bosentan on mean arterial pressure and renal blood flow

There was no significant difference between mean arterial pressure in GalN animals and normal controls (106 (5) *v* 115 (4) mmHg) (table 3). There was however a significant reduction in renal blood flow in GalN rats compared with controls (1.8 (0.2) *v* 2.9 (0.2) ml/min/kidney) (p<0.0001). This corresponds to 0.52 ml/min/100 g and 0.83 ml/min/100 g for GalN rats and sham rats, respectively. In a separate series of experiments, Bosentan was infused into the femoral vein of rats with GalN induced liver failure, and renal blood flow was monitored. Bosentan had no effect on renal blood flow (2.0 (0.4) ml/min/kidney) compared with GalN alone (1.8 (0.2) ml/min/kidney). Renal vascular resistance was significantly increased in rats with GalN induced liver failure compared with controls (69 (9) *v* 44 (4) mm Hg/ml/min) (p<0.05), and this was unchanged following injection of Bosentan (renal vascular resistance 72 (15) mm Hg/ml/min) (table 3).

DISCUSSION

The development of acute liver failure following administration of GalN to rats is well documented.[22–24] Hepatocyte necrosis occurs secondary to inhibition of hepatic RNA synthesis through formation of UDP hexosamines,[22] thus inhibiting gene transcription. As a consequence of liver failure, this model develops several features commonly observed in patients with acute liver failure—namely, cerebral oedema and coagulopathy, and a hyperdynamic circulation.[23 24] In this study we have shown that there is a significant reduction in creatinine clearance and an increase in serum creatinine. However, despite the development of renal failure, histologically the kidneys were normal. Moreover, there were no electron microscopic features of acute tubular necrosis. The only abnormality observed in the kidney on electron microscopy examination was a minor increase in vacuolation in the proximal tubules of GalN treated animals, the cause and significance of which are unknown. Electron microscopy studies in patients with functional renal failure have previously demonstrated proximal tubular necrosis, tubulorrhexis, and mitochondrial dark bodies[31] although these changes were considerably more extensive than those observed in this study. Indeed, in one of the original descriptions of this model, it was stated that there was no histological evidence of tissue damage in organs other than the liver.[22] The modest increase in urinary volume observed following the onset of acute liver failure might suggest that mild tubular injury has occurred, and which is not evident on light microscopy. The presence of increased vacuolation in the proximal tubules observed by electron microscopy may therefore take on a new significance. However, the kidneys retained the ability to concentrate urine with a threefold concentration above plasma osmolality, and there was no significant change in free

water clearance. The observation that urinary sodium excretion decreased in animals with acute liver and renal failure suggests that tubular function is preserved but it may also be due to decreased food intake by animals with liver failure. The absence of any direct toxic effect of GalN on cultured $LLCPK_1$ cells, a renal tubular cell line, also suggests that GalN is not directly nephrotoxic. Moreover, concentrations of GalN employed in the in vitro studies (up to 50 mM) far exceed those present in vivo, which are known to be directly toxic to hepatocytes in culture.[32] The most compelling data that seems to exclude the possibility that GalN is acting as a nephrotoxic agent are the observations that when Bosentan is given 24 hours following GalN, it still protects against the development of renal impairment (fig 3). However, data from this study do not exclude the possibility that GalN may cause mild renal tubular injury, which may be independent of that causing decreased GFR.

The observation that there was a marked reduction in renal blood flow in this model confirms the findings of Javle and colleagues.[24] This occurs despite an increase in cardiac output and systemic vasodilatation, as observed in other studies.[23] The significant reduction in renal blood flow and development of a hyperdynamic circulation in this model parallels similar changes observed in patients with acute liver failure who develop renal failure.[3 6 33] The reduction in renal blood flow occurred independent of any significant change in arterial blood pressure, and was secondary to renal vasoconstriction. Under normal circumstances, reduced renal perfusion is accompanied by a decrease in salt and water excretion. In this study, there was a modest increase in urine output, such that fractional excretion of sodium was slightly but significantly increased.

At present there are no ideal animal models of HRS. Models of renal dysfunction secondary to liver disease include the bile duct ligated rat, rabbit, dog, and baboon[34–36] in which renal blood flow is decreased. However, most workers regard the acute bile duct ligated rat as a model of cholestasis and endotoxaemia rather than of HRS. Both Zambraski *et al* and Levy *et al* have shown that dog models of chronic bile duct ligation will develop renal failure in the absence of haemodynamic changes when these animals are given a cyclo-oxygenase inhibitor.[37 38] Other models include the Long-Evans Cinnamon strain of rat, which is characterised by increased storage of copper in the liver and kidney and the development of renal abnormalities.[39] Whether this is due to direct toxicity of the copper is not yet clear. Recently studies on kidney function have been carried out in carbon tetrachloride induced cirrhotic rats. These animals develop cirrhosis after 2–3 months of chronic administration of carbon tetrachloride, and subsequently develop ascites with salt and water retention, and a slight decrease in GFR.[40] In many respects the carbon tetrachloride model resembles that of cirrhosis, with salt and water retention, and modest changes in renal function. However, such studies have to be interpreted in the light of the extensive peroxidation of renal lipids that occurs in this model. Thus Morrow and colleagues have demonstrated that following administration of carbon tetrachloride, there was a 50-fold increase in the levels of esterified F_2 isoprostanes in the kidney, indicative of lipid peroxidation.[41] While this tends to be acute—that is, for a few hours following carbon tetrachloride administration—the presence of renal injury by carbon tetrachloride will always raise questions as to whether altered renal function is secondary to liver injury or due to renal injury.

The GalN model shares many similarities with patients with acute liver failure, and this includes the development of renal failure that occurs in many patients with acute liver failure. It does however differ in one important aspect—namely, that there is increased urine output rather than oliguria that usually occurs in patients with acute liver failure and renal failure. While there is resistance to the idea that patients with acute liver and renal failure have HRS, this group were specifically included in the definition agreed at a consensus conference held at the American Association for the Study of Liver Disease in 1994.[5] The current data would support the use of this animal model for renal failure that occurs in acute liver failure and possibly alcoholic hepatitis, in which rapid and acute decompensation of liver function occurs. It clearly cannot be regarded as a model for HRS that develops in patients with end stage cirrhosis and ascites.

We have previously shown that patients who develop HRS, particularly in the context of acute liver failure or alcoholic hepatitis, have increased circulating concentrations of ET-1.[19] Further evidence to support a role for ET-1 in the pathogenesis was that administration of BQ123, an ET_A selective antagonist, improved renal function in four patients with HRS.[42] However, no further studies using ET-1 receptor antagonists have been carried out in humans, and the role of ET-1 in the pathogenesis of HRS is still conjectural. Kramer *et al* demonstrated that rats with acute biliary obstruction and renal dysfunction had increased circulating ET-1 concentrations, and that administration of Bosentan (a combined ET_A and ET_B receptor antagonist) improved renal function.[43] In the current study, a twofold increase in plasma ET-1 and significant upregulation of the ET_A receptor in the renal cortex were observed in animals with acute liver and renal failure. These data, together with the findings that Bosentan prevents the development of renal failure, confirm that ET-1 has an important role as a determinant of renal function in this model of acute liver and renal failure. The mechanisms leading to increased circulating ET-1 concentrations are unknown. ET-1 is synthesised by a multitude of cell types, including endothelial, hepatic stellate, mesangial, and vascular smooth muscle cells, as well as a variety of epithelial cells. Our observation that tissue levels of ET-1 are highest in the renal medulla is consistent with previous studies.[44] The mechanisms leading to increased synthesis of ET-1 in liver disease are unknown but have been postulated to involve endotoxaemia, vasodilatation and sheer stress, activation of hepatic stellate cells, and a response to effective hypovolaemia.[19 43]

One surprising aspect of these studies was the observation that infusion of Bosentan did not increase renal blood flow acutely. This may occur for one of two reasons. Firstly, ET-1 may bind avidly with its receptor, and is not easily or rapidly displaced by Bosentan at the doses used. Secondly, it may be that ET-1 exerts it action on kidney function at the microvascular level, by causing contraction of mesangial cells within the glomeruli and thus decreasing the surface area available for glomerular filtration. Thus ET-1 may cause a reduction in GFR over and above that caused by the reduction in renal blood flow alone, and dissociate the changes in renal blood flow from those of GFR.

In summary, the current study demonstrates that the GalN model of acute liver failure shares many of the characteristics needed to be regarded as a model of type I HRS. Renal failure appears to be secondary to liver failure in that the kidneys are histologically normal and renal blood flow is decreased. ET-1 is important in the pathogenesis of renal failure as there is an increase in plasma ET-1 concentrations, upregulation of the ET_A receptor in the renal cortex, and administration of an ET-1 receptor antagonist improves renal function. We conclude that ET-1 has a central role in the pathogenesis of the renal dysfunction that occurs in this model of acute liver failure.

ACKNOWLEDGEMENTS

This work was funded by the Medical Research Council, UK, and the Peter Samuel Research Fund. We would like to thank Dr Roger Ling (Medicine), J Lewin (Electron Microscope Unit, RFH), Francis Moll (Histopathology), David Blundell (Autonomic Neuroscience Institute), and John Muddle (Neurological Sciences) for advice and help with these studies.

Authors' affiliations
R Anand, D Harry, S Holt, K Moore, Department of Medicine, Royal Free and University College Medical School, London, UK
D Goodier, Department of Clinical Chemistry, Royal Free Hospital, London, UK
M Jarmulowicz, Department of Histopathology, Royal Free Hospital, London, UK
M Dashwood, Department of Molecular Pathology, Clinical Biochemistry and Surgery, Royal Free and University College Medical School, London, UK
P Milner, The Autonomic Neuroscience Institute, Royal Free and University College Medical School, London, UK

REFERENCES

1 **Shear L**, Kleinerman J, Gabuzda GJ. Renal failure of patients with cirrhosis of the liver. I. Clinical and pathological characteristics. *Am J Med* 1965;**39**:184–90.
2 **Epstein M**. Hepatorenal syndrome. In: Epstein M, ed. *The kidney in liver disease*, 4th edn. Philadelphia: Hanley & Belfus, Inc., 1996:75–108.
3 **Ring-Larsen H**, Palazzo U. Renal failure in fulminant hepatic failure and terminal cirrhosis: a comparison between incidence, types and prognosis. *Gut* 1981;**22**:585–91.
4 **Wilkinson SP**, Blendis IK, Williams R. Frequency and type of renal and electrolyte disorders in fulminant hepatic failure. *BMJ* 1974;**i**:186–9.
5 **Arroyo V**, Gines P, Gerbes AL, *et al*. Definition and diagnostic criteria of refractory ascites and hepatorenal syndrome in cirrhosis. *Hepatology* 1996;**23**:164–76.
6 **Guarner F**, Hughes RD, Gimson AE, *et al*. Renal function in fulminant hepatic failure: haemodynamics and renal prostaglandins. *Gut* 1987;**28**:1643–7.
7 **Epstein M**, Berck DP, Hollemberg NK, *et al*. Renal failure in the patient with cirrhosis. The role of active vasoconstriction *Am J Med* 1970;**49**:175–85.
8 **Ring-Larsen H**. Renal blood flow in cirrhosis: relation to systemic and portal haemodynamics and liver function. *Scand J Clin Lab Invest* 1977;**37**:635–42.
9 **Badr KF**, Murray JJ, Breyer MD, *et al*. Mesangial cell, glomerular and renal vascular resistance response to endothelin in the rat kidney. Elucidation of signal transduction pathways. *J Clin Invest* 1989;**83**:336–42.
10 **Yanagisawa M**, Kurihara H, Kimura S, *et al*. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 1988;**332**:411–15.
11 **Firth JD**, Raine AE, Ratcliffe PJ, *et al*. Endothelin: an important factor in acute renal failure. *Lancet* 1988;**19**:1179–81.
12 **Cairnes HS**, Rogerson ME, Fairbanks LD, *et al*. Endothelin induces an increase in renal vascular resistance and a fall in glomerular filtration rate in the rabbit isolated perfusion kidney. *Br J Pharmacol* 1989;**98**:155–60.
13 **Lopez-Farre A**, Montanes I, Millas I, *et al*. Effect of endothelin on renal function in rats. *Eur J Pharmacol* 1989;**163**:187–9.
14 **Sorensen SS**, Madsen JK, Pedersen EB. Systemic and renal effect of intravenous infusion of endothelin-1 in healthy human volunteers. *Am J Physiol* 1994;**266**:F411–8.
15 **Uchida S**, Takemoto F, Ogata E, *et al*. Detection of endothelin-1 mRNA by CT-PCR in isolated rat renal tubules. *Biochem Biophys Res Commun* 1992;**188**:108–13.
16 **Sakamoto H**, Sasaki S, Hirata Y, *et al*. Production of endothelin-1 by rat cultured mesangial cells. *Biochem Biophys Res Commun* 1990;**169**:462–8.
17 **Nambi P**, Wu H-L, Pullen M, *et al*. Identification of endothelin receptor subtypes in rat kidney cortex using subtype-selective ligands. *Mol Pharmacol* 1992;**42**:336–9.
18 **Kohan DE**, Hughes AK, Perkins SL. Characterization of ET receptors in the inner medullary collecting duct of the rat. *J Biol Chem* 1992;**267**:12336–40.
19 **Moore K**, Wendon J, Frazer M, *et al*. Plasma endothelin immunoreactivity in liver disease and the hepatorenal syndrome. *N Engl J Med* 1992;**327**:1774–8.
20 **Uemasu J**, Matsumoto H, Kawasaki H. Increased plasma endothelin levels in patients with liver cirrhosis. *Nephron* 1992;**60**:380–4.
21 **Moller S**, Emmeluth C, Henriksen JH. Elevated circulating plasma endothelin-1 concentrations in cirrhosis. *J Hepatol* 1993;**19**:285–90.
22 **Keppler D**, Lesch R, Reutter W, *et al*. Experimental hepatitis induced by D-galactosamine. *Exp Mol Pathol* 1968;**9**:279–90.
23 **Makin AJ**, Hughes RD, Williams R. Systemic and hepatic hemodynamic changes in acute liver injury. *Am J Physiol* 1997;**272**:G617–25.
24 **Javle P**, Yates J, Kynaston HG, *et al*. Hepatosplanchnic hemodynamics and renal blood flow and function in rats with liver failure. *Gut* 1998;**43**:272–9.
25 **Clozel M**, Breu V, Gray GA, *et al*. Pharrmacological characterization of bosentan, a new potent orally active nonpeptide endothelin receptor antagonist. *J Pharm Exp Ther* 1994;**270**:228–35.
26 **Bodin P**, Milner P, Marshall J, *et al*. Cytokines suppress the shear stress-stimulated release of vasoactive peptides from human endothelial cells. *Peptides* 1995;**16**:1433–8.
27 **Dashwood MR**, Sykes RM, Collins MJ, *et al*. Autoradiographic localization of [^{125}I]endothelin binding sites in human blood vessels and coronary tissue: functional correlates. *Neurochem Int* 1990;**18**:439–44.
28 **Davenport AP**, Kuc RE, Fitzgerald F, *et al*. [125I]-PD151242: a selective radioligand for human ETA receptors. *Br J Pharmacol* 1994;**111**:4–6.
29 **Molenaar P**, Kuc RE, Davenport AP. Characterization of two new ETB selective radioligands, [125I]-BQ3020 and [125I]-[Ala 1,3,11,15]ET-1 in human heart. *Br J Pharmacol* 1992;**107**:637–9.
30 **Welch WJ**, Deng X, Snellen H, *et al*. Validation of miniature ultrasonic transit-time flow probes for measurement of renal blood flow in rats. *Am J Physiol* 1995;**268**:F175–8.
31 **Mandal AK**, Lansing M, Fahmy A. Acute tubular necrosis in hepatorenal syndrome: an electron microscopy study. *Am J Kidney Dis* 1982;**2**:363–74.
32 **Wu J**, Soderbergh H, Karlsson K, *et al*. Protective effect of S-adenosyl-L-methionine on bromobenzene-and D-galactosamine-induced toxicity to isolated rat hepatocytes. *Hepatology* 1996;**23**:359–65.
33 **Epstein M**, Schneider N, Befeler B. Relationship of systemic and intrarenal haemodynamics in cirrhosis. *J Lab Clin Med* 1977;**89**:1175–87.
34 **Bloom DS**, Bomzon L, Rosendorff C, *et al*. Renal blood flow in obstructive jaundice: an experimental study in baboons. *Clin Exp Pharmacol Physiol* 1976;**3**:461–72.
35 **Hishida A**, Honda N, Sudo M, *et al*. Mechanisms of altered renal perfusion in the early stage of obstructive jaundice. *Kidney Int* 1980;**17**:223–30.
36 **Better OS**, Massry SG. Effect of chronic bile duct obstruction on renal handling of salt and water. *J Clin Invest* 1972;**51**:402–11.
37 **Zambraski EJ**, Dunn MJ. Importance of renal postaglandins in control of renal function after chronic ligation of the common bile duct in dogs. *J Lab Clin Med* 1984;**103**:549–59.
38 **Levy M**, Wexler MJ, Fechner C. Renal perfusion in dogs with experimental hepatic cirrhosis: role of prostaglandins. *Am J Physiol* 1983;**245**:F521–9.
39 **Nomiyama K**, Nomiyama H, Kameda N, *et al*. Mechanism of hepatorenal syndrome in rats of Long-Evans Cinnamon strain, an animal model of fulminant Wilson's disease. *Toxicology* 1999;**132**:201–14.
40 **Martin PY**, Ohara M, Gines P, *et al*. Nitric oxide synthase (NOS) inhibition for one week improves renal sodium and water excretion in cirrhotic rats with ascites. *J Clin Invest* 1998;**101**:235–42 .
41 **Morrow JD**, Awad JA, Kato T, *et al*. Formation of novel non-cyclooxygenase-derived prostanoids (F2-isoprostanes) in carbon tetrachloride hepatotoxicity. An animal model of lipid peroxidation. *J Ciin Invest* 1992;**90**:2502–7.
42 **Soper PR**, Latif AB, Bending MR. Amelioration of hepatorenal syndrome with selective endothelin-A antagonist. *Lancet* 1996;**347**:1842–3.
43 **Kramer HJ**, Schwarting K, Backer A, *et al*. Renal endothelin system in obstructive jaundice: its role in impaired renal function of bile-duct ligated rats. *Clin Sci* 1997;**92**:579–85.
44 **Wilkes BM**, Myron S, Mento PF, *et al*. Localization of endothelin-like immunoreactivity in rat kidneys. *Am J Physiol* 1991;**260**:F913–20.