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Comparison of endothelin receptors in normal versus cirrhotic human liver and in the liver from endothelial cell-specific ET_B knockout mice

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ABSTRACT

Aims: Endothelin (ET) antagonists show promise in animal models of cirrhosis and portal hypertension. The aim was to pharmacologically characterise the expression of endothelin receptors in human liver, hepatic artery and portal vein.

Main methods: Immunofluorescence staining, receptor autoradiography and competition binding assays were used to localise and quantify ET receptors on hepatic parenchyma, hepatic artery and portal vein in human cirrhotic or normal liver. Additional experiments were performed to determine the affinity and selectivity of ET antagonists for liver ET endothelin receptors. An endothelial cell ET_B knockout murine model was used to examine the function of sinusoid endothelial ET_B receptors.

Key findings: ET_B receptors predominated in normal human liver and displayed the highest ratio (ET_B:ET_A 63:47) compared with other peripheral tissues. In two patients examined, liver ET_B expression was up-regulated in cirrhosis (ET_B:ET_A 83:17). Both sub-types localised to the media of normal portal vein but ET_B receptors were downregulated fivefold in the media of cirrhotic portal vein. Sinusoid diameter was fourfold smaller in endothelial cell ET_B knockout mice. The liver morphology of ET_B knockout mice was markedly different to normal murine liver, with loss of the wide spread sinusoidal pattern. In the knockout mice, sinusoids were reduced in both number and absolute diameter, while large intrahepatic veins were congested with red blood cells.

Significance: These data support a role for the ET system in cirrhosis of the liver and suggest that endothelial ET_B blockade may cause sinusoidal constriction which may contribute to hepatotoxicity associated with some endothelin antagonists.

Introduction

Current medical treatment of portal hypertension (PH) is inadequate and patient mortality remains high resulting from complications of chronic liver disease, including variceal haemorrhage. The aim of therapy and prophylaxis is to lower portal pressure to levels that reduce the risk of variceal haemorrhage and death. Meta-analysis has demonstrated that a portal pressure of less than 12 mmHg or a greater than 20% reduction from baseline portal pressure reduces variceal bleeding and mortality (D'Amico et al., 2006). Current pharmacological interventions, including beta-blockade, aim to reduce splanchnic flow and portal pressure, but 30–40% of patients either do not respond or target reduction in portal pressure is not achieved (Garcia-Tsao et al., 1986; Merkel et al., 2000). Liver transplantation is an effective treatment for both cirrhosis and PH, but the demand for donor organs continues to outstrip supply. With an increasing prevalence of chronic liver disease and limited treatment options, there is clinical urgency for new medical therapy.

A role for the endothelin (ET) system in liver cirrhosis has been suggested by clinical studies that demonstrated a correlation between the severity of cirrhosis and elevated plasma endothelin-1 (ET-1) levels (Tsai et al., 1995; Uchihara et al., 1992). ET-1 mediates its actions via two receptors, ET_A and ET_B (Davenport, 2002; Davenport and Maguire, 2006) and in healthy rat liver, both subtypes are concentrated along sinusoids, mainly on hepatic stellate cells and sinusoidal endothelial cells (Gondo et al., 1993; Housset et al., 1993). In rats with cirrhosis, expression of both receptors on hepatic stellate cells was reported to be up-regulated, but receptor

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expression on sinusoidal endothelial cells was unchanged (Yokomori et al., 2001a). Using immunohistochemistry, ET_B expression was also reported to be up-regulated in human cirrhotic liver, with low ET_A expression detected in both normal and diseased liver (Yokomori et al., 2001b). Endothelin may also be an important regulator of sinusoid vascular resistance mediated by vasoconstriction of stellate cells, the main effector of sinusoid calibre (Rockey and Weisiger, 1996). In animal studies infusion of ET-1 caused a dose-dependent decrease in sinusoid diameter (Okumura et al., 1994; Zhang et al., 1994) and both selective ET_A and mixed antagonists were shown to decrease portal pressure (Feng et al., 2009; Sogni et al., 1998). Additionally, ET antagonists may also decrease the fibrotic response in cirrhosis (Khimji and Rockey, 2011).

Our aim was to determine the distribution and density of ET receptors in human normal and cirrhotic liver parenchyma, hepatic artery and portal vein. Immunofluorescence staining and autoradiography were used to localise and quantify ET receptors on normal and diseased hepatic artery and portal vein. Receptor autoradiography and competition binding assays were used to quantify ET receptors and to determine the binding characteristics of ET antagonists in human liver. Lastly, an endothelial cell specific ET_B knockout murine model was used to investigate the consequence of loss of ET_B receptors to provide evidence that selective ET_A antagonism may be the treatment of choice in cirrhosis and PH.

Materials and methods

BQ123, cyclo[D-Asp-L-Pro-D-Val-L-Leu-D-Trp-], BQ788, (N-cis-2,6-dimethylpiperidinocarbonyl-γL-MeLeu-D-Trp(CooMe)-D-Nle-ONa) and BQ3020, [$Ala^{11,\ 15}$]Ac-ET-$1_{6\text{-}21}$ were synthesised by solid phase t-Boc chemistry. [^{125}I]-ET-1 was from PerkinElmer, while unlabelled ET-1 was from Peptide Institute. Ambrisentan and sitaxentan were from Gilead Sciences, Inc. and Pfizer, Inc., respectively. ET_A and ET_B rabbit antisera were raised to the C-terminus of ET_A(413–427) and to ET_B(302–313) (Davenport and Kuc, 2005a). Primary goat anti-von Willebrand factor and anti-smooth muscle α-actin were from Dako; secondary fluorescent antibodies were from Invitrogen.

Human tissue samples

Tissue samples collected at the time of operation were obtained with ethical approval (REC 10/H0305) and informed consent. Diseased liver parenchyma, hepatic artery and portal vein were obtained from patients undergoing liver transplantation for non-infectious causes of end-stage liver disease. Normal liver parenchyma, hepatic artery and portal vein samples were from donor liver and vessels that were not used for transplantation surgery or were normal tissue from liver resections. Unless otherwise stated *n*-values refer to the number of patients from whom tissue was obtained.

Endothelial cell specific ET_B knockout mice

Mice were generated using the Cre-loxP system (Bagnall et al., 2006). After euthanasia, consecutive, cryostat-cut frozen sections (10 μm) of knock-out and control mouse torsos were mounted onto gelatine coated glass microscope slides. Sinusoid diameter in ET_B knockout and control mice were measured under light microscopy. A two-sided unpaired *t*-test was used to compare changes in sinusoid number and between control and knock-out mice, with significance set at $P<0.05$.

Dual-labelled immunofluorescence microscopy

Methods were as previously described (Davenport and Kuc, 2005a). Briefly, tissue sections (10 μm) were dried overnight at room temperature and fixed in ice-cold acetone for 10 minutes. Slides were incubated with 5% non-immunised donkey serum (DS) in phosphate-buffered saline (PBS) for 1 hour at room temperature to block non-specific protein interactions and then incubated overnight at 4 °C with primary rabbit anti-ET_A (1:50) or anti-ET_B (1:50) antiserum and either primary goat anti-von Willebrand factor (1:50) or goat anti-smooth muscle α-actin (1:100) antibody diluted in 1% PBS/0.1% Tween-20/3% DS. Slides were then washed (3×5 minutes) in cold 1% PBS/0.1% Tween-20 before incubation for 1 hour at room temperature with Alexa Fluor 488 conjugated donkey anti-rabbit (1:200), Alexa Fluor 568 conjugated donkey anti-goat (1:100) secondary antibodies and Hoechst (1:100) diluted in 1% PBS/0.1% Tween-20/3% DS. Tissue sections were washed again (3×5 minutes) in cold 1% PBS/0.1% Tween-20 and mounted with ProLong Gold (Invitrogen). Confocal imaging was performed using a Leica TCS-NT-UV confocal laser-scanning microscope (Leica Microsystems, Heidelberg, Germany).

Quantitative autoradiography

Adjacent 10 μm tissue sections were incubated with HEPES buffer (50 mM HEPES, 5 mM MgCl2, 0.3% bovine serum albumin, pH 7.4) for 20 minutes at room temperature. Sections were incubated for 2 hours at room temperature with [^{125}I]-ET-1 (0.1 nM) alone for total binding and either with unlabelled ET-1 (1 μM) to determine non-specific binding, with BQ3020 (100 nM) to label ET_A receptors or with BQ123 (100 nM) to label ET_B receptors (Molenaar et al., 1993). Sections were washed (3×5 minutes) in ice-cold Tris–HCl buffer (50 mM, pH 7.4), air dried and apposed, together with [^{125}I]-ET-1 standards, to Kodak MR-1 autoradiography film for 2 days at room temperature. The resulting autoradiographs were analysed using computer-assisted densitometry (Quantimet 970, Leica, Milton Keynes, UK) (Davenport and Kuc, 2005b) and receptor densities expressed in amol/mm^2.

Competition binding assays

Adjacent 10 μm tissue sections were incubated with HEPES buffer (50 mM HEPES, 5 mM MgCl2, 0.3% bovine serum albumin, pH 7.4) for 20 minutes at room temperature and then for 2 hours at room temperature with [^{125}I]-ET-1 (0.1 nM) and either sitaxentan (20 pM to 200 nM), BQ788 (20 pM to 200 nM) or ambrisentan (20 pM to 200 nM). Non-specific binding was determined by inclusion of 1 μM ET-1. Sections were washed (3×5 minutes) in ice-cold Tris–HCl buffer (50 mM, pH 7.4) and the amount of tissue bound [^{125}I]-ET-1 measured by gamma counting. Data were analysed using EBDA software (McPherson, 1983) to provide initial estimates of equilibrium dissociation constant (K_D) and maximum binding density (B_{max}) and LIGAND software (Munson and Rodbard, 1980) to determine final estimates based on the *F*-ratio test ($P<0.05$) of 1, 2 or 3 site models. The B_{max} was normalised to protein concentration (Davenport and Kuc, 2005b). Results were expressed as the mean ± standard error of the mean.

Results

Dual-labelled immunofluorescence microscopy

Confocal photomicrographs of a transverse section through a human portal vein from a patient with cirrhosis are shown in Fig. 1A and B. Antisera to ET_A (Fig. 1A) was visualised as green fluorescence and a second antisera to the smooth muscle marker α-actin was visualised in red. Co-localization of both antisera within the medial and intimal smooth muscle layers of portal vein is shown in yellow. In Fig. 1B, antisera to ET_B was visualised as green fluorescence and α-actin in red, showing that both sub-types were detectable on smooth muscle. ET_A and ET_B immunoreactivity was also localised to the adventitial layer of

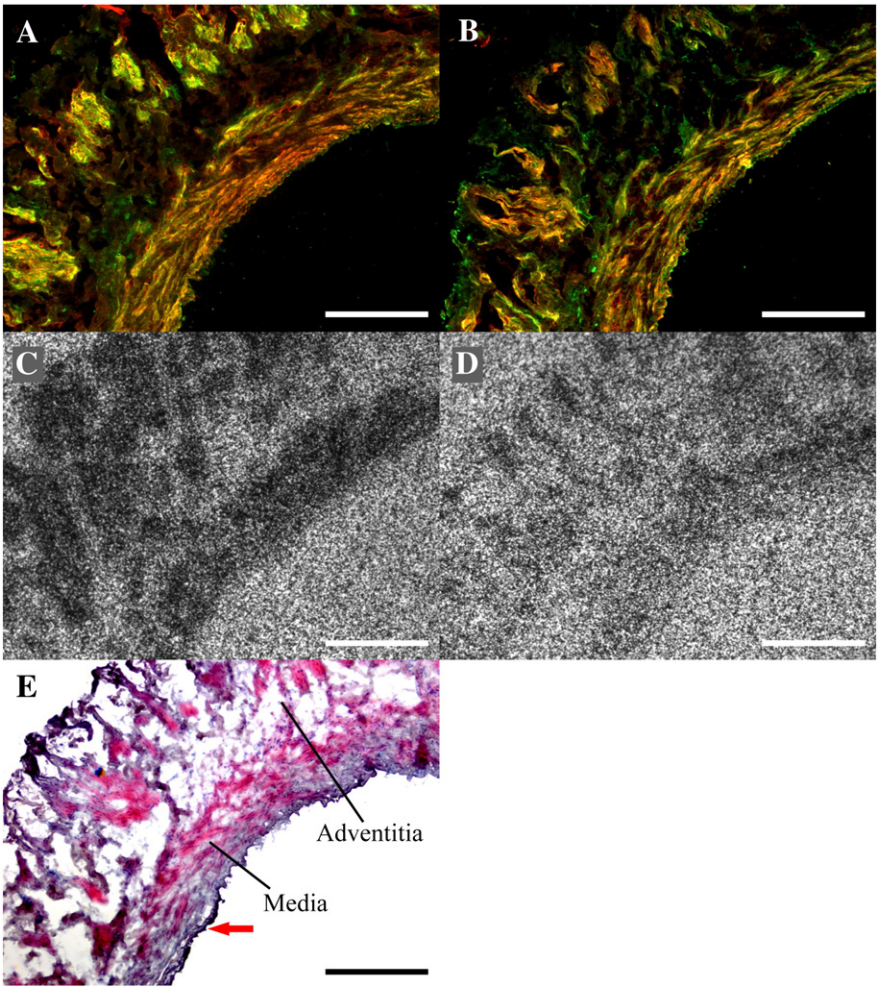

Fig. 1. (A) Digitally overlaid confocal photomicrographs illustrating the co-localization (shown in yellow) of antisera to ET_A or ET_B (B) (shown in green) and the smooth muscle marker α-actin (shown in red) in a transverse section of human portal vein from a patient with cirrhosis. In the corresponding autoradiographs for the distribution of ET_A (C) and ET_B (D), binding of the radioligand is shown in black. (E) Adjacent section stained with haematoxylin and eosin comparative histology shows the endothelium (red arrow), media and adventitia. Scale bars represent 500 μm.

both vessels. Results at the cellular level were supported by radioligand binding using [^{125}I]-ET-1 where the distribution of ET_A (C) and ET_B (D) receptors is shown by the blackening of autoradiography film. Adjacent sections stained with haematoxylin and eosin were used to delineate the endothelium (red arrow), media and adventitia.

Confocal photomicrographs (Fig. 2) illustrating the localization of antisera to ET_A (A, shown in green) the endothelial cell marker vWF (B), shown in red to visualise the single layer of cells in the vascular endothelium in a transverse section of human portal vein from a patient with cirrhosis. Digitally overlaid confocal photomicrographs (C) illustrating the co-localization are shown in yellow. Corresponding images of ET_B immunoreactivity are shown in (Fig. 2D), vWF (Fig. 2E) and the digital overlay demonstrating co-localization in Fig. 2F.

Autoradiography

In normal liver parenchyma, there was a homogenous distribution of ET receptors within the tissue, measured by radioligand binding and quantitative autoradiography. Total ET receptor density measured using [^{125}I]-ET-1 which binds with the same affinity to both sub-types was 152 ± 11 amol/mm^2 with ET_A receptors comprising 41 ± 6 amol/mm^2 ($37 \pm 5\%$) and ET_B receptors 81 ± 23 amol/mm^2, ($63 \pm 5\%$) (Fig. 3A and B). In preliminary studies using tissue from two patients with cirrhosis the total receptor density was reduced to about 100 amol/mm^2 and the ratio of ET_A:ET_B was 17:83. Therefore,

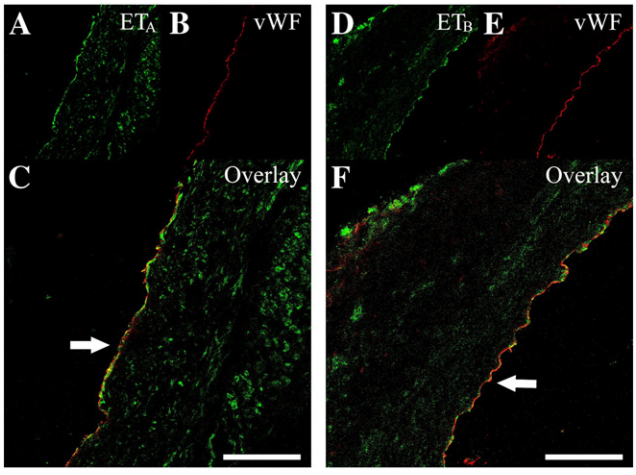

Fig. 2. Dual labelled immunofluorescence confocal imaging of ET_A and ET_B in cirrhosis human portal vein (A and B). ET receptors (green) are co-localised (yellow) with von Willebrand factor (vWF, red). Specific ET receptor staining is seen on the endothelium (white arrow).

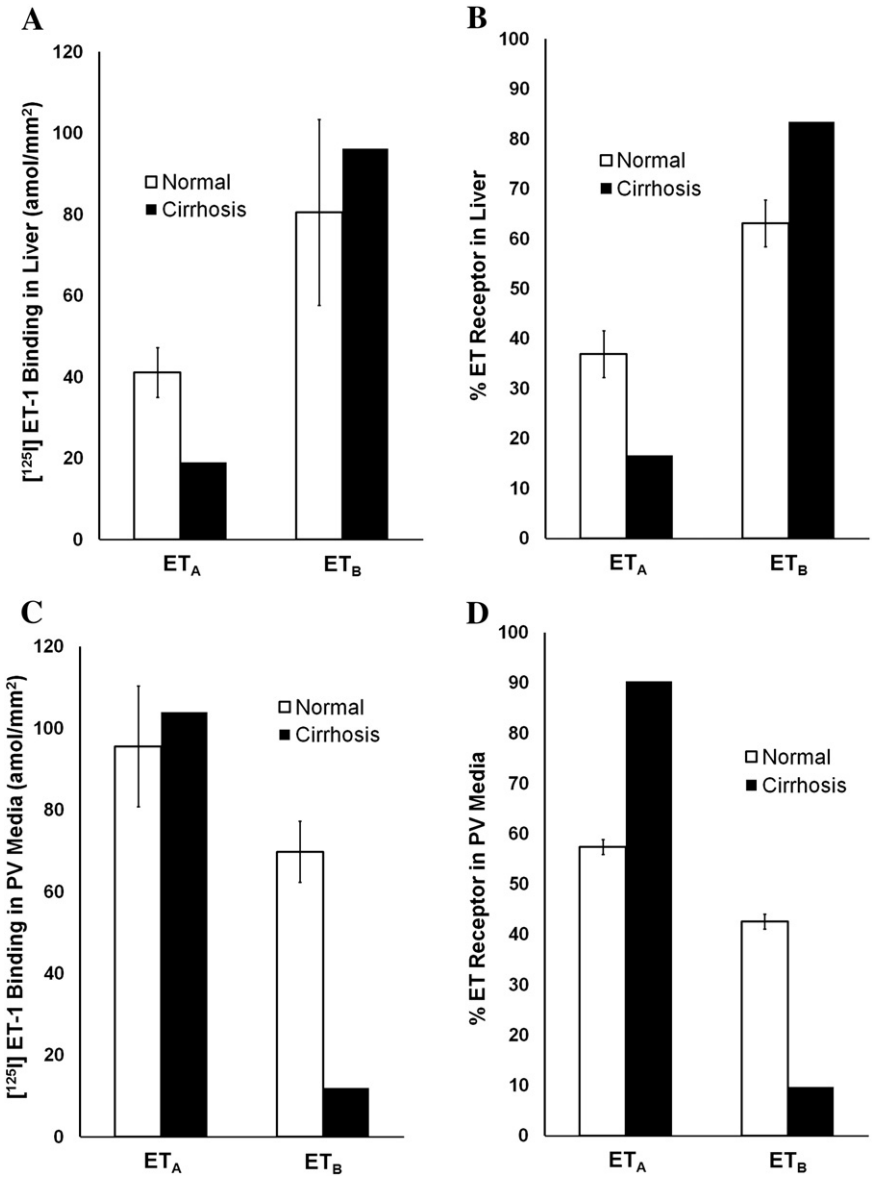

Fig. 3. (A) Density of [^{125}I]-ET-1 binding to ET_A and ET_B in human liver parenchyma and (B) expressed as a ratio of each sub-type. There was a shift in the ratio ET_A:ET_B from 37:63 in normal liver to 17:83 in cirrhotic livers. (C) [^{125}I]-ET-1 binding to ET_A and ET_B receptors in the medial layer of portal vein and (D) expressed as a ratio. There was also a change in diseased media from 57.4% ET_A and 42.6% ET_B to 90.3% ET_A and 9.7% ET_B. Each value represents mean and standard error of the mean.

in liver parenchyma, cirrhosis was associated with a downregulation of ET_A (19 amol/mm^{-2}) and small increase in ET_B (96 amol/mm^{-2}) receptor density (Fig. 3A and B). Cirrhotic liver parenchyma had a characteristic nodular pattern with ET_A localised mainly in the fibrous septa and at lower levels within lobules. Similarly, ET_B was also concentrated along the fibrous connective tissue between lobules in the diseased liver, but ET_B binding was also high within the lobules (Fig. 4).

Autoradiographs revealed the expression of ET_A and ET_B receptors determined using binding of [^{125}I]-ET-1 in portal vein (Fig. 1C and D) and hepatic artery were consistent with that obtained by immunohistochemistry. In normal portal vein ET receptor density was 149 ± 25 amol/mm^2 in the medial smooth muscle layer with an ET_A:ET_B ratio of 57:43 (96 ± 15 amol/mm^2 ET_A and 70 ± 8 amol/mm^2 ET_B). In portal vein from patients with cirrhosis the overall density of ET receptors was lower (131 amol/mm^2) but the ratio of ET_A:ET_B was 90:10. Therefore, in contrast to parenchyma, in portal vein there was no change in medial ET_A receptor expression but there was a downregulation of ET_B receptors (Fig. 3C and D).

Competition binding

The results of the ability of the three antagonists, BQ788, sitaxentan and ambrisentan to compete for the binding of [^{125}I]-ET-1 are shown in Fig. 5. Pooling data from competition binding curves for the three antagonists, BQ788, sitaxentan and ambrisentan (Fig. 5) total ET receptor density were not different in cirrhotic liver (150.0 ± 22.7 fmol/mg) compared to normal liver (125.6 ± 12.2 fmol/mg). The ET_B receptor comprised the predominant subtype in normal liver and the proportion of ET_B was increased further in disease with a shift in ET_A:ET_B ratio from 20:80 in normal tissue to 5:95 with cirrhosis. As expected, the ET_B selective antagonist, BQ788 exhibited high affinity for the ET_B receptor in both normal (K_D 37 ± 8 nM) and diseased (133 ± 34 nM) liver with lower micromolar affinity for the ET_A receptor (normal ET_A K_D 65 ± 88 μM; cirrhosis ET_A K_D 46 ± 108 μM). Since the majority of the ET_B receptors in liver were of the ET_B subtype, the density of ET_A receptors in normal and particularly cirrhotic liver was too low to derive an accurate affinity constant at the ET_A receptor for sitaxentan and ambrisentan. However, from the competition curves it

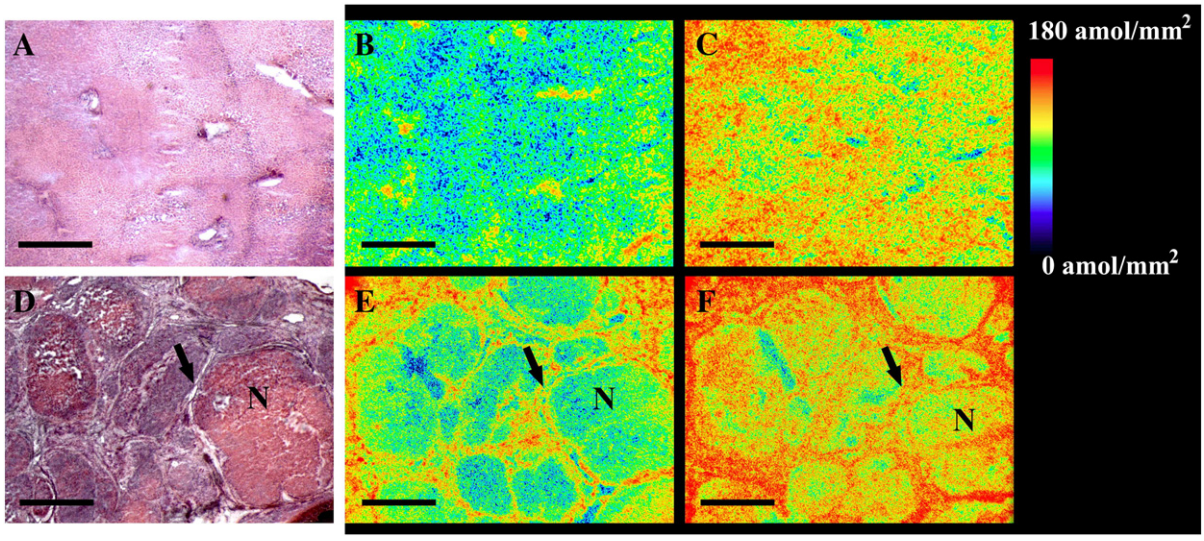

Fig. 4. (A) Haematoxylin and eosin staining of a transverse section of normal and (D) cirrhotic liver, showing regenerative nodules (N) and fibrous septa (black arrow). (B) Colour-coded autoradiograph showing the distribution of [^{125}I]-ET-1 binding to ET_A and (C) ET_B in human normal liver. (E) ET_A and (F) ET_B in cirrhosis liver parenchyma. Scale bars represents 1 mm.

was clear that both compounds competed for the ET_B receptors in the low micromolar range.

Endothelial cell specific ET_B knockout mice

Livers from endothelial cell ET_B knockout mice were examined and compared to normal murine liver under light microscopy (Fig. 6). The liver morphology of ET_B knockout mice was markedly different to normal murine liver, with loss of the wide spread sinusoidal pattern seen in normal livers. In the knockout mice, sinusoids were reduced in both number and absolute diameter, while large intrahepatic veins were congested with red blood cells. Sinusoidal diameter was reduced from 22.3 ± 1.5 μm in normal murine liver to 6.0 ± 0.4 μm in knockout mice ($P = 0.0004$).

Discussion

Studies on the ET pathway in cirrhosis have thus far focused on the liver parenchyma itself (Ikura et al., 2004). This study characterises ET receptors for the first time in human portal vein. Consistent with studies on other human vessels, we found that smooth muscle cell ET_A is the main receptor subtype in the media of hepatic artery and portal vein (Bacon and Davenport, 1996; Davenport et al., 1995). As there was a six fold decrease in ET_B density in portal vein media, the ET_A: ET_B ratio in portal vein media changed from 57:43 in health to 90:10 in cirrhosis. It is possible that elevated plasma ET-1 in cirrhosis coupled with relatively high ET_A expression in portal vein media may increase pre-hepatic portal resistance and cause PH. Therefore, ET_A antagonism may be beneficial in dilating the portal vein in PH. It should be emphasised that these are preliminary descriptive studies and the results justify further experiments to determine reproducibility in a larger number of individuals.

Immunofluorescence staining for ET_A and ET_B was found along the endothelium of normal and cirrhosis hepatic artery and portal vein. While it is well established that ET_B is present on the endothelium of human vessels, endothelial ET_A is less well documented (Nishimura et al., 1995). Endothelial ET_B mediates NO release from the endothelium, and is an important counter mechanism to ET-1 induced vasoconstriction on smooth muscle ET_A (Hirata et al., 1993; Honoré et al., 2002). Moreover, endothelial ET_B functions as clearing receptors that remove excess ET-1 from the circulation (Johnstrom

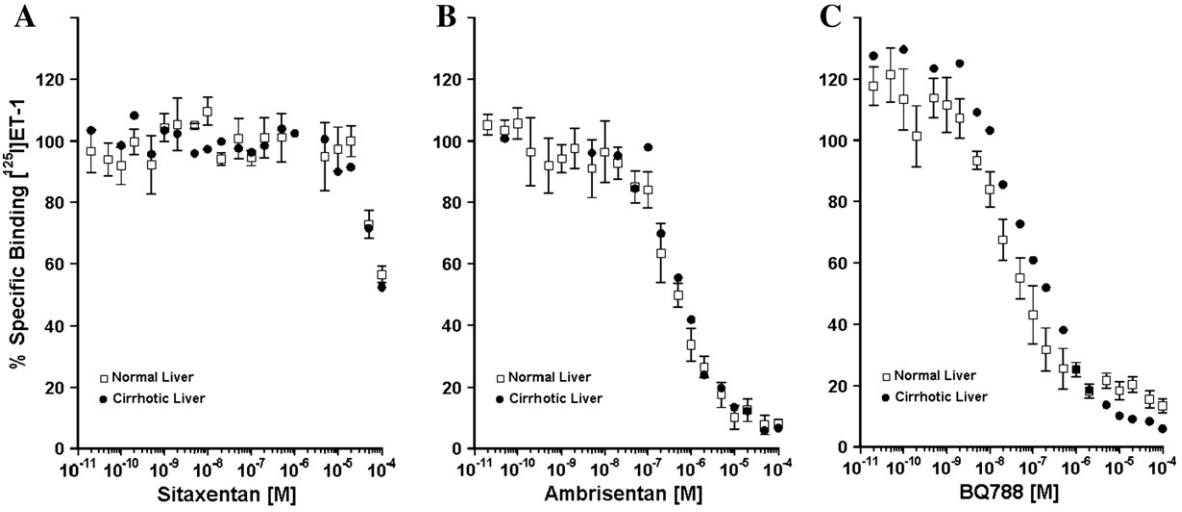

Fig. 5. (A) Competition binding of [^{125}I]-ET-1 against sitaxentan, (B) ambrisentan and (C) BQ788 in human normal and cirrhotic liver. Each value represents mean and standard errors of the mean.

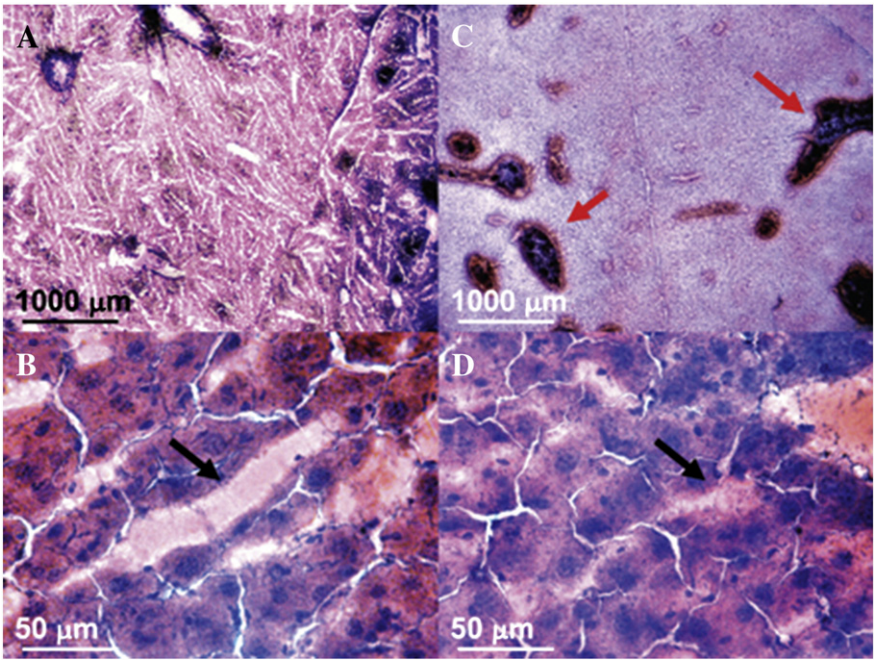

Fig. 6. (A) Brightfield image of normal (A and B) and endothelial cell ET_B knockout mice (C to D) livers using haematoxylin and eosin staining. Sinusoid (black arrow) diameter is reduced in knockout mice and results in lost of the normal sinusoidal pattern on low magnification. In the knockout mice, intrahepatic branches of the portal vein are congested with blood (red arrow).

et al., 2005; Kelland et al., 2010). While ET_A antagonism may be beneficial in PH, whether ET_B antagonism is also helpful is less straight forward, as the opposing functions of endothelial ET_B (dilatation and reducing plasma ET-1) and smooth muscle ET_B (constriction) complicate therapeutic choice. Nevertheless, animal studies showed that mixed antagonists such as bosentan are as effective as ET_A selective antagonists in reducing portal pressure (Feng et al., 2009; Rockey and Weisiger, 1996). The choice between selective ET_A and mixed antagonism continues to be debated in the treatment of pulmonary arterial hypertension (Vachiéry and Davenport, 2009) and renal failure (Davenport and Maguire, 2011).

Interestingly sinusoid diameter was reduced three fold in the knockout compared to wild type mice and intrahepatic branches of the portal vein were congested with red blood cells (Fig. 6). This suggests that blood was unable to pass through sinusoids effectively owing to reduced sinusoidal diameter and produced congestion in the portal venous system. Hence, sinusoidal endothelial cell ET_B may be critical in maintaining adequate sinusoidal diameter. Therefore, ET_B blockade which causes sinusoid constriction and consequent liver ischaemia may contribute to the liver toxicity observed with mixed antagonists. This suggests additional benefit of ET_A selective compounds.

This study has identified changes in ET receptor ratio and density within human cirrhotic liver and portal vein. ET receptor densities from quantitative autoradiography and competition binding experiments are consistent with those suggested by previous studies on the human liver (Yokomori et al., 2001b). Both techniques demonstrated a change towards upregulation of ET_B and downregulation of ET_A in the cirrhotic liver. The significance of these changes is undetermined, but it may be a physiological response towards normalising elevated portal pressure in cirrhosis. Adding the results from this study to data from animal models, selective ET_A antagonism may be a beneficial therapy for cirrhosis and PH by four distinct mechanisms. First, it may reduce prehepatic portal resistance by dilatation of the portal vein. Second, it may decrease intrahepatic resistance by reducing hepatic stellate cells contraction and increasing sinusoid diameter. Third, it may also reduce intrahepatic resistance by reversing fibrosis and restoring normal liver architecture. Fourth, it preserves the beneficial sinusoidal endothelial cell ET_B mediated dilatation and clearance of elevated plasma ET-1. Further in vitro studies using human tissues are required with antagonists of the propionic acid class of compounds (such as ambrisentan) that do not display significant hepatotoxicity but as our results show are ET_A selective in human liver, for the treatment of cirrhosis.

Conflict of interest statement

N.J.D. was an employee of Pfizer. The research was supported in part by an investigator led grant from Pfizer.

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