Angiotensin-(1–7), an alternative metabolite of the renin–angiotensin system, is up-regulated in human liver disease and has antifibrotic activity in the bile-duct-ligated rat

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ABSTRACT

Ang-(1–7) (angiotensin-1–7), a peptide product of the recently described ACE (angiotensin-converting enzyme) homologue ACE2, opposes the harmful actions of AngII (angiotensin II) in cardiovascular tissues, but its role in liver disease is unknown. The aim of the present study was to assess plasma levels of Ang-(1–7) in human liver disease and determine its effects in experimental liver fibrosis. Angiotensin peptide levels were measured in cirrhotic and non-cirrhotic patients with hepatitis C. The effects of Ang-(1–7) on experimental fibrosis were determined using the rat BDL (bile-duct ligation) model. Liver histology, hydroxyproline quantification and expression of fibrosis-related genes were assessed. Expression of RAS (renin–angiotensin system) components and the effects of Ang-(1–7) were examined in cirrhotic and non-cirrhotic patients with hepatitis C. The effects of Ang-(1–7) on experimental fibrosis were determined using the rat BDL (bile-duct ligation) model. Liver histology, hydroxyproline quantification and expression of fibrosis-related genes were assessed. Expression of RAS (renin–angiotensin system) components and the effects of Ang-(1–7) were examined in cirrhotic and non-cirrhotic patients with hepatitis C. In human patients with cirrhosis, both plasma Ang-(1–7) and AngII concentrations were markedly elevated (P < 0.001). Non-cirrhotic patients with hepatitis C had elevated Ang-(1–7) levels compared with controls (P < 0.05), but AngII concentrations were not increased. In BDL rats, Ang-(1–7) improved fibrosis stage and collagen Picrosirius Red staining, and reduced hydroxyproline content, together with decreased gene expression of collagen 1A1, α-SMA (smooth muscle actin), VEGF (vascular endothelial growth factor), CTGF (connective tissue growth factor), ACE and mas [the Ang-(1–7) receptor]. Cultured HSCs expressed AT1Rs (AngII type 1 receptors) and mas receptors and, when treated with Ang-(1–7) or the mas receptor agonist AVE 0991, produced less α-SMA and hydroxyproline, an effect reversed by the mas receptor antagonist A779. In conclusion, Ang-(1–7) is up-regulated in human liver disease and has antifibrotic actions in a rat model of cirrhosis. The ACE2/Ang-(1–7)/mas receptor axis represents a potential target for antifibrotic therapy in humans.

Key words: angiotensin-(1–7), angiotensin-converting enzyme, cirrhosis, hepatic fibrosis, renin–angiotensin system.

Abbreviations: ACE, angiotensin-converting enzyme; ACEI, ACE inhibitor; ALP, alkaline phosphatase; ALT, alanine aminotransaminase; AngII, angiotensin II; Ang-(1–7), angiotensin-(1–7); AT1R, AngII type 1 receptor; ARB, AT1R blocker; BDL, bile-duct ligation (ligated); CHC, cirrhotic patient with hepatitis C; CTGF, connective tissue growth factor; GGT, γ-glutamyl transpeptidase; HBSS, Hanks balanced salt solution; HSC, hepatic stellate cell; MELD, Model for End-stage Liver Disease; NHC, non-cirrhotic patient with hepatitis C; Q-PCR, quantitative PCR; RAS, renin–angiotensin system; SMA, smooth muscle actin; VEGF, vascular endothelial growth factor.

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INTRODUCTION

Ang-(1–7) (angiotensin 1–7) is an alternative metabolite of the RAS (renin–angiotensin system) with actions that oppose those of AngII (angiotensin II). Interest in Ang-(1–7) has been invigorated by the discovery of the ACE (angiotensin-converting enzyme) homologue ACE2 [1,2]. This novel enzyme, although similar in structure to ACE, has distinct enzyme activity, and catalyses the conversion of AngII into Ang-(1–7) [3]. Although ACE2 is capable of generating Ang-(1–7) indirectly through the intermediary peptide Ang-(1–9) and subsequent enzymatic cleavage by ACE, in vitro evidence suggests this pathway is less important [4].

Ang-(1–7) has a number of important actions, including vasodilatation [5,6] and inhibition of cell proliferation [7–9], as well as anti hypertensive [10] and anti arrhythmic [11] effects. In addition, Ang-(1–7) has been implicated in the protection of tissues from chronic injury through direct effects on tissue fibrogenesis [8,12]. The effects of Ang-(1–7) appear to be mediated through the mas receptor [13], although other undefined receptor subtypes may well exist [14]. The ACE2/Ang-(1–7)/mas receptor axis therefore appears to represent a counter-regulatory arm of the RAS capable of protecting chronically damaged tissues from the harmful effects of locally produced AngII [15].

Despite considerable evidence supporting the beneficial effects of Ang-(1–7) in renal and cardiovascular disease, the effects of this peptide have not been studied in the liver. We have shown previously [16] that key components of the classical RAS are markedly up-regulated in chronic liver injury. More recently, we have shown that ACE2 gene and tissue activity are up-regulated by chronic liver injury in humans and the BDL (bile-duct-ligated) rat, and that Ang-(1–7) and mas receptor expression increase as liver injury progresses [17,18]. These studies suggest that in liver disease the ACE2/Ang-(1–7)/mas receptor axis is up-regulated in response to the effects of chronic damage, including activation of the ACE/AngII/AT1R (AngII type 1 receptor) arm of the RAS. Support for a possible protective role of Ang-(1–7) in liver disease has been provided by an in vivo animal experiment in which the mas receptor was pharmacologically inhibited [19]. However, to date, there has been no direct evidence that administration of the Ang-(1–7) peptide can influence hepatic fibrosis in animal models nor has there been confirmation that circulating levels of this peptide are increased in human liver disease. In this present study, we therefore measured circulating Ang-(1–7) and AngII plasma concentrations in cirrhotic patients with hepatitis C (CHC group) awaiting transplantation and compared them with non-cirrhotic patients with hepatitis C (NHC group) and healthy controls. Furthermore, we have examined the effect of Ang-(1–7) infusion on the fibrotic response to BDL in the rat and in cultured rat HSCs (hepatic stellate cells). The BDL model was chosen to determine whether Ang-(1–7) can moderate the injurious effects of AngII because it is a robust model of cholestatic disease, in which there is up-regulation of both classical and novel components of the RAS [17]. In BDL rats, serum AngII concentration are markedly increased [16], and AngII infusion exacerbates liver injury and bile-duct proliferation [20].

This research has future clinical implications since both Ang-(1–7) and a non-peptide orally active mas receptor agonist, AVE 0991, may constitute a new therapeutic option for the treatment of fibrotic diseases [21].

MATERIALS AND METHODS

Human observational study

Patient recruitment

Patients with chronic hepatitis C virus and control subjects (n = 54 total) provided written informed consent for the present study, which was designed in accordance with the Declaration of Helsinki (2000) and was approved by the Austin Health Human Ethics Committee. The CHC group (n = 9) were recruited from the liver transplant waiting list, and the NHC group (n = 23) were recruited in an outpatient setting. The absence of cirrhosis in these patients was confirmed by liver biopsy, and the severity of liver fibrosis ranged from METAVIR fibrosis stage 0 to 3 (median, 2). Exclusion criteria included renal impairment (serum creatinine > 120 μmol/l), prior haemofiltration or haemodialysis in the preceding 1 month or significant cardiovascular disease, including hypertension. Patients were also excluded if they were taking medication known to influence the RAS [ACEIs (ACE inhibitors), ARBs (AT1R blockers) and β-blockers]. Control subjects for blood analysis were healthy volunteer healthcare professionals (n = 13), and healthy liver samples for gene expression data were taken from patients (n = 9) without liver fibrosis who were undergoing hepatic resection for non-hepotoma-related lesions. Five out of the patients in the CHC group subsequently underwent liver transplantation and samples of their explanted livers were snap frozen in liquid nitrogen prior to subsequent gene analysis.

Blood analysis

Blood was taken from subjects in the seated position. Blood for RIAs of angiotensin peptides was collected in a tube containing an enzyme inhibitor mix (50 mmol/l EDTA, 21,000 units/ml aprotinin and 0.2 mol/l N-ethylmalainide). All samples were immediately mixed, placed on ice and centrifuged. Plasma and serum samples were stored at −20°C until the time of assay. The serum ACE activity assay was performed with
a commercial method utilizing the hydrolysis of a synthetic substrate 3-(2-furylacryloyl)-L-phenylalanylglyclylglycine (Beckman Coulter Uncilic DXX 800 analyser). RIAs for AngII and Ang-(1–7) were performed on all plasma samples in duplicate (ProSearch International), as described previously [18]. Briefly, the RIAs for Ang-(1–7) and AngII were performed using an antibody raised in guinea-pig to Ang-(1–7) N-terminally conjugated to porcine thyroglobulin and Ang-(1–7) mono-iodinated with 125I, and an antibody raised in rabbit to AngII N-terminally conjugated with BSA and AngII mono-iodinated with 125I. The antibody-bound 125I-Ang-(1–7) was separated from free peptide by secondary antibody absorption. The intra- and inter-assay coefficients of variation for Ang-(1–7) and AngII were 4.5 and 10 % respectively.

**ACE2 gene expression**

ACE2 hepatic gene expression was assessed in all five patients from the CHC group who underwent liver transplantation and were compared with healthy liver tissue from nine patients undergoing hepatic resection. Gene expression was quantified as outlined below.

**Animal interventional study**

**Experimental design**

Male Sprague–Dawley rats were housed in a controlled environment (12 h light/12 h dark; temperature, 22–24 °C), and fed standard rat chow (Norco) with water ad libitum. All animal experiments were conducted according to the National Health and Medical Research Council (NHMRC) Guidelines for Animal Experimentation, and the experimental protocol was approved by the Austin Health Animal Ethics Committee.

Biliary hepatic fibrosis was induced in 6-week-old male Sprague–Dawley rats by double ligation and transection of the common bile duct, as described previously [16,22]. Sham-operated animals acted as a control group. All rats were anaesthetized with an intraperitoneal injection of a ketamine/xylazine mixture (70 and 7 mg/kg of body weight respectively; Therapon) and were given a single dose of carprofen (2 mg/kg of body weight; Lyppard) subcutaneously prior to surgery in order to limit post-operative discomfort. Animals were divided into three groups each containing 11 rats. Osmotic pumps designed to deliver their contents over 2 weeks were implanted into the abdominal cavity of all animals (Model 2002; Alzet). Sham-operated rats received osmotic pumps containing saline. Osmotic pumps containing either saline or Ang-(1–7) (28 μg·kg⁻¹·h⁻¹ of body weight·h⁻¹; Auspep) were implanted into BDL rats. The dose and mode of delivery of Ang-(1–7) was based on previous studies using osmotic pumps delivering similar concentration of Ang-(1–7) within the peritoneal cavity [23]. Animals were killed 2 weeks after surgery under lethal anaesthesia induced by sodium pentobarbital (60 mg/kg of body weight), and blood samples were taken for liver function tests. Body, liver and spleen weights were recorded, and tissue samples were taken from liver, snap frozen in liquid nitrogen and stored at –80 °C until assayed for gene expression and hydroxyproline content. Tissue sections were also collected in 4 % paraformaldehyde for histological and immunohistochemical evaluations. Osmotic pumps were collected at the time of killing and the volumes of any residual contents were recorded.

**Biochemical and histological assessment of liver injury and fibrosis**

Plasma bilirubin, ALT (alanine aminotransaminase), ALP (alkaline phosphatase) and GGT (γ-glutamyl transpeptidase) were measured on a Beckman Coulter LX20 autoanalyser.

Sections of liver (4 μm) mounted on silane-coated glass slides were stained with haematoxylin and eosin and Picrosirius Red (Polysciences). Liver sections were assessed in random order by an experienced liver pathologist, who was blinded to the animal groups. Sections were assessed for METAVIR fibrosis score and the ductal proliferation score, as adapted from Miyoshi et al. [24]. The number of biliary infarcts was also documented for each field examined.

Collagen content of the liver was quantified using a hydroxyproline colorimetric assay and computerized quantification of Picrosirius Red staining, as described previously [25,26]. Picrosirius Red staining was assessed at ×100 magnification in a total of ten fields per animal, and was performed in a blinded fashion using computerized image capture (MCID; Imaging Research). Results are expressed as relative Picrosirius Red staining normalized to the sham + saline group, which was given the mean value of 1.

**α-SMA (smooth muscle actin) immunohistochemistry**

Immunohistochemical staining for α-SMA, a marker for activated HSCs, was performed on 4 μm sections of paraffin-embedded rat liver tissue-mounted on silane-coated glass slides. Specimens were de-waxed in histolene and dehydrated in graded ethanol, and endogenous peroxidase activity was removed by treating sections with 3 % H₂O₂ in PBS for 20 min. Non-specific proteins were blocked with protein-blocking agent (Lipshaw Immunon) diluted in distilled water. The primary antibody to α-SMA (monoclonal 1A4; DakoCytomation) at a dilution of 1:25 in 0.1 % normal goat serum with PBS was applied and the slides were left overnight at 4 °C. The following day, sections were allowed to reach room temperature (18–22 °C) and were incubated with a biotinylated-conjugated secondary goat anti-(mouse IgG) antibody at
a dilution of 1:100, followed by incubation with avidin–biotin horseradish peroxidase. Peroxidase conjugates were subsequently localized using DAB (diaminobenzidine; Sigma). The relative staining in each group was determined by computerized quantification (MCID) in a total of ten fields per animal at ×100 magnification.

**In vitro ACE autoradiography**

Frozen liver sections were cut to a thickness of 20 μm using a cryostat (Microtome Cryostat HM 550; MICROM) at −20 °C, and specimens were thaw-mounted on to slides. A specific radioligand, 125I-MK351A, was used for the ACE autoradiography as described previously [16]. Semi-quantitative analysis of the binding density was performed using computerized densitometry (MCID).

**Q-PCR (quantitative PCR)**

Q-PCR reactions were carried out using multiplexing with amplification of both the target gene and endogenous reference gene occurring in a single well and were analysed as described previously [18]. The details of dual-fluorescent labelled oligonucleotide probes and primers are given in Supplementary Table 1 (available at http://www.ClinSci.org/cs/117/cs1170375add.htm).

**HSC study**

HSCs were isolated from rat livers as described previously [27]. Briefly, livers from Sprague–Dawley rats (n = 3) weighing 400–500 g were perfused with HBSS (Hanks balanced salt solution; Invitrogen), followed by a solution containing 150 mg of pronase and 15 mg of collagenase A (Roche Diagnostics). Livers were removed and mechanically dispersed by gently mixing with 300 mg of pronase, 30 mg of collagenase A and 10 mg of Dnase (Roche Diagnostics) made up to a total volume of 100 ml of HBSS. This mixture was agitated constantly in an incubator at 37°C for 45 min and filtered through a 100 μm nylon mesh to remove any undigested debris. The cell suspension underwent a series of centrifugation steps before density gradient separation with Nycodenz (Sigma). The total cell yield and viability was assessed by counting the number of dead and live cells, based on Trypan Blue exclusion. Cellular purity was confirmed by staining of cytospin preparations for the typical HSC markers desmin (Dako), vimentin (Santa Cruz Biotechnology) and glial fibrillary acidic protein (Chemicon International). Purity was assessed further by typical morphological features (vitamin A autofluorescence) and by staining of cytospin preparations for the typical HSC study.

**Patient demographics**

The characteristics of patients and control subjects are shown in Table 1. As expected, control subjects were generally younger than in the NHC group, who were in turn younger than those in the CHC group, and there were more males in the patient groups compared with the control group. The patients were older than those in the NHC group, who were generally younger than the patient groups.

**RESULTS**

**Human observational study**

**Statistics**

Results are means ± S.E.M., unless otherwise stated. A Gaussian distribution was tested using the Kolmogorov–Smirnov test. Normally distributed data were analysed by ANOVA, with a Newman–Keuls multiple comparison test and a two-tailed unpaired Student’s t test, where appropriate. Non-parametric data were analysed using the Kruskal–Wallis test and Dunn’s multiple comparison test. The assumption of equal variance was tested using Bartlett’s test. Data sets with unequal variance were transformed to approximate the distribution to a Gaussian distribution. A P value of <0.05 was considered statistically significant. Statistical analyses were performed using GraphPad Prism 4 software for Macintosh.

**RESULTS**

**Human observational study**

**Patient demographics**

The characteristics of patients and control subjects are shown in Table 1. As expected, control subjects were generally younger than in the NHC group, who were in turn younger than those in the CHC group, and there were more males in the patient groups compared with the control group. Previous analyses of control subjects has demonstrated that AngII and Ang-(1–7) plasma levels are independent of either age or gender (J. S. Lubel, C. B. Herath, Z. Jia, D. Casley and P. W. Angus, unpublished work).
Table 1  Characteristics of the patients and controls

*P < 0.05, **P < 0.01 and ***P < 0.001 compared with the CHC group; †P < 0.05, ††‡P < 0.01 and †††P < 0.001 compared with the control group. RR, reference range.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control (n = 13)</th>
<th>NHC (n = 23)</th>
<th>CHC (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender (n) (male/female)</td>
<td>7/6</td>
<td>15/8</td>
<td>7/2</td>
</tr>
<tr>
<td>Bilirubin (RR, &lt; 18.6 µmol/l)</td>
<td>12.7 ± 0.6</td>
<td>18.6 ± 1.2***†††</td>
<td>61.2 ± 10.3***†††</td>
</tr>
<tr>
<td>ALP (RR, 32–91 units/l)</td>
<td>67.4 ± 6.3</td>
<td>75.6 ± 4.5***</td>
<td>116.9 ± 14.7***†††</td>
</tr>
<tr>
<td>ALT (RR, &lt; 45 units/l)</td>
<td>13.4 ± 1.7</td>
<td>112.2 ± 18.2††‡†</td>
<td>43.0 ± 9.3††</td>
</tr>
<tr>
<td>GGT (RR, &lt; 55 units/l)</td>
<td>30.5 ± 13.4</td>
<td>89.4 ± 28.0††‡†</td>
<td>54.7 ± 8.2†††</td>
</tr>
<tr>
<td>MELD score</td>
<td>6.4 ± 0.18</td>
<td>7.9 ± 0.34***††</td>
<td>16.3 ± 1.16†††</td>
</tr>
</tbody>
</table>

Table 2  Circulating concentrations of ACE, AngII and Ang-(1–7) levels and Ang-(1–7)/AngII ratio in patients and controls

*P < 0.01 and ***P < 0.001 compared with the CHC group; †P < 0.05 and †††P < 0.001 compared with the control group.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Control</th>
<th>NHC</th>
<th>CHC</th>
</tr>
</thead>
<tbody>
<tr>
<td>AngII (pg/ml)</td>
<td>19.61 ± 2.39</td>
<td>23.36 ± 2.56***</td>
<td>192.9 ± 49.19†††</td>
</tr>
<tr>
<td>Ang-(1–7) (pg/ml)</td>
<td>15.95 ± 0.35</td>
<td>34.77 ± 10.39††</td>
<td>35.36 ± 6.98†</td>
</tr>
<tr>
<td>Ang-(1–7)/AngII</td>
<td>1.24 ± 0.29</td>
<td>1.92 ± 0.41***</td>
<td>0.33 ± 0.11†††</td>
</tr>
<tr>
<td>ACE (units/l)</td>
<td>31.23 ± 4.08</td>
<td>64.04 ± 5.55†††</td>
<td>129.4 ± 29.14†††</td>
</tr>
</tbody>
</table>

Plasma AngII and Ang-(1–7) levels

AngII plasma concentrations were significantly elevated in the CHC group compared with the NHC and control groups (P < 0.01 and P < 0.001 respectively; Table 2). However, there was no significant difference in AngII concentrations between the control and NHC groups. Furthermore, there was no correlation between age or gender and peptide concentrations in all of the groups studied. In contrast, plasma Ang-(1–7) levels were significantly (P < 0.05) elevated in both the CHC and NHC groups compared with the control group (Table 2). In the CHC group, both AngII and Ang-(1–7) plasma levels failed to show any correlation with disease severity [MELD (Model for End-stage Liver Disease) score] or biochemical markers of liver injury. The Ang-(1–7)/AngII molar ratio was very low in the CHC group compared with both the NHC and control groups (P < 0.001), whereas the NHC group had a similar molar ratio to that of the control group (Table 2).

Serum ACE activity levels

Serum ACE levels were significantly elevated in the CHC group compared with both the control and NHC groups (P < 0.01), and the level in the NHC group was also higher than those in the control group (P < 0.001; Table 2). There was no correlation between age and ACE level in any of the groups.

Hepatic ACE2 mRNA expression in human liver

ACE2 gene expression was increased more than 3-fold in the livers from the CHC group compared with healthy liver (3.450 ± 1.272 compared with 1.000 ± 0.273; P < 0.05), confirming that this key regulator of Ang-(1–7) production in the alternative axis of the RAS is up-regulated in response to hepatic injury.

Animal interventional study

At the end of the 2 week period, all pumps had minimal (10–30 µl) residual content from the original volume of 200 µl, confirming that the contents had been appropriately delivered.

Body and organ weights

Initial body weights were similar in all three groups (Table 3). Following sham operation, rats continued to thrive and gained weight at a rate of 12% of initial body weight/week. Liver and spleen weights were significantly (P < 0.001) elevated in both of the BDL groups, indicating that BDL resulted in hepatosplenomegaly (Table 3). There was no significant difference in spleen or liver weight between the two BDL groups. Only minimal ascites was present in the BDL rats.

Ang-(1–7) reduced histological fibrosis and hepatic hydroxyproline content

Both BDL groups had significantly (P < 0.001) elevated liver function tests compared with the sham-operated group, confirming that BDL had been successfully performed; however, Ang-(1–7) infusion resulted in significantly (P < 0.05) improved serum bilirubin and GGT (Table 3). BDL resulted in significant histological changes, including expansion of portal tracts with extensive bile duct proliferation. There were also numerous regions...
of biliary infarction and extensive fibrosis with bridging and nodule formation (Figures 1A and 1B).

Treatment with Ang-(1–7) resulted in a significant ($P < 0.05$) reduction in METAVIR and ISHAK fibrosis scores and bile-duct proliferation score compared with the BDL + saline group ($n = 11$ each group; Table 3). There was no difference in the number of biliary infarcts observed in the two BDL groups.

The hepatic hydroxyproline content of the BDL + saline group was increased more than 2-fold compared with the sham-operated group ($P < 0.05$). Infusion of Ang-(1–7) reduced this increase in hydroxyproline by 33% ($P < 0.05$; Table 3). These changes in hepatic collagen were confirmed by proportional area assessment of Picosiris Red staining, which demonstrated that Ang-(1–7) infusion reduced the relative Picosiris Red staining from that observed in the BDL + saline group by more than 50% ($P < 0.001$; Figure 1B and Table 3).

### Ang-(1–7) reduced α-SMA staining

Increased α-SMA staining was observed in livers of BDL rats (Figures 1C and Figure 2). When the two BDL treatment groups were compared, there was a reduction in the staining for α-SMA cells in the Ang-(1–7)-infusion group compared with the BDL + saline group ($P < 0.05$; Figure 1C and Figure 2). This result suggests that Ang-(1–7) attenuates fibrosis by suppression of HSC activation.

### Ang-(1–7) reduced hepatic ACE as assessed by in vitro ACE autoradiography

Sham-operated animals had a low level of ACE radioligand binding, which increased 350% in the BDL + saline group (Figure 3). However, this was significantly ($P < 0.001$) reduced in the BDL + Ang-(1–7) group to a level approaching that of the sham group (133%; Figure 3).

### Q-PCR

The hepatic gene expressions of key RAS components, together with markers of fibrosis and inflammation, are shown in Figure 4.

Gene expression of ACE2 was increased more than 30-fold following BDL ($P < 0.001$), with minimal difference between the two BDL groups. ACE gene expression increased 55-fold in the BDL + saline group ($P < 0.001$), but, in contrast with ACE2, ACE gene expression was significantly ($P < 0.05$) reduced by Ang-(1–7) infusion. There was a small, but significant, increase in AT1R gene expression as a result of BDL ($P < 0.05$) reduced by Ang-(1–7) infusion. mas was up-regulated in the BDL + saline group by approx. 10-fold with Ang-(1–7) infusion,

#### Table 3  Animal characteristics, liver function tests, hepatic collagen content and bile-duct proliferation scores

Relative Picosiris Red staining was normalized to the sham + saline group, which is given a mean value of 1.* $P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ compared with the BDL + saline group (ANOVA); † $P < 0.05$, †† $P < 0.01$ and ††† $P < 0.001$ compared with the sham + saline group.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Sham + saline ($n = 11$)</th>
<th>BDL + saline ($n = 11$)</th>
<th>BDL + Ang-(1–7) ($n = 11$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight (g)</td>
<td>265.3 ± 5.5</td>
<td>243.1 ± 10.2</td>
<td>236.4 ± 10.8</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>330.9 ± 8.7</td>
<td>262.6 ± 12.1†††</td>
<td>276.6 ± 9.5†††</td>
</tr>
<tr>
<td>Spleen weight (g)</td>
<td>0.82 ± 0.05</td>
<td>1.62 ± 0.07†††</td>
<td>1.54 ± 0.08†††</td>
</tr>
<tr>
<td>Spleen index (% of body weight)</td>
<td>0.249 ± 0.016</td>
<td>0.625 ± 0.033†††</td>
<td>0.555 ± 0.031†††</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>14.70 ± 0.402</td>
<td>18.90 ± 0.612†††</td>
<td>19.42 ± 0.567†††</td>
</tr>
<tr>
<td>Liver index (% of body weight)</td>
<td>4.446 ± 0.076</td>
<td>7.275 ± 0.243†††</td>
<td>7.001 ± 0.181†††</td>
</tr>
<tr>
<td>Liver function test</td>
<td></td>
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<tr>
<td>ALT (units/l)</td>
<td>51.70 ± 1.687</td>
<td>138.2 ± 11.80†††</td>
<td>151.9 ± 13.84†††</td>
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<tr>
<td>ALP (units/l)</td>
<td>275.4 ± 12.39</td>
<td>463.0 ± 31.90†††</td>
<td>437.1 ± 33.10†††</td>
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<tr>
<td>GGT (units/l)</td>
<td>5.22 ± 0.13</td>
<td>47.55 ± 5.64†††</td>
<td>32.64 ± 5.74†††</td>
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<tr>
<td>Bilirubin (μmol/l)</td>
<td>5.50 ± 0.31</td>
<td>143.9 ± 13.67†††</td>
<td>104.6 ± 12.15†††</td>
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<tr>
<td>Hepatic collagen</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>ISHAK fibrosis score</td>
<td>0.6 ± 0.152</td>
<td>5.5 ± 0.207†††</td>
<td>4.5 ± 0.282**†††</td>
</tr>
<tr>
<td>METAVIR fibrosis score</td>
<td>0.0 ± 0.0</td>
<td>3.409 ± 0.113†††</td>
<td>2.864 ± 0.195**†††</td>
</tr>
<tr>
<td>Relative Picosiris Red staining</td>
<td>1.0 ± 0.134</td>
<td>8.17 ± 0.720†††</td>
<td>3.74 ± 0.619***†††</td>
</tr>
<tr>
<td>Hydroxyproline content (μg/g)</td>
<td>151.6 ± 31.80</td>
<td>385.4 ± 46.93†††</td>
<td>259.6 ± 13.03††</td>
</tr>
<tr>
<td>Bile-duct proliferation</td>
<td></td>
<td></td>
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<tr>
<td>Ductal proliferation score</td>
<td>0.0 ± 0.0</td>
<td>3.82 ± 0.182†††</td>
<td>2.91 ± 0.211***†††</td>
</tr>
</tbody>
</table>
Role of Ang-(1–7) in human and experimental liver disease

Figure 1  Representative photomicrographs of livers from the sham + saline, BLD + saline and BDL + Ang-(1–7) groups
Paraffin-embedded sections were stained with haematoxylin and eosin (A), Picrosirius Red (B) and α-SMA (C).

Figure 2  Quantification of α-SMA immunohistochemistry in the sham + saline, BLD + saline and BDL + Ang-(1–7) groups
n = 11 in each group.

HSC study

HSC activation results in the increased expression of both AT₁R and mas receptor
Both the AT₁R and mas receptor, the key target receptors for AngII and Ang-(1–7) respectively, were expressed in HSCs. At 7 days of culture, there was marked increased expression of both AT₁R and mas receptor gene expression compared with quiescent HSCs [AT₁R, 6.296 ± 1.199 compared with 1.000 ± 0.193 respectively (P < 0.05); mas receptor, 4.639 ± 1.485 compared with 1.000 ± 0.451 respectively (P < 0.05)], indicating that both axes of the RAS are up-regulated by HSC activation.

Ang-(1–7) and AVE0991 reduce the cellular expression of α-SMA
In keeping with our findings in vivo, Ang-(1–7) treatment reduced HSC activation, as shown by a dose-dependent decrease in α-SMA concentration (P < 0.005, as determined by ANOVA with a post-test for the linear trend; Figure 5). The greatest effect on α-SMA was observed with the Ang-(1–7) agonist AVE0991, which

resulting in a significant down-regulation of this gene compared with the BDL + saline group (P < 0.01).

Collagen 1A1, α-SMA, CTGF (connective tissue growth factor) and VEGF (vascular endothelial growth factor) genes were significantly (P < 0.05) up-regulated by BDL. Importantly, infusion of Ang-(1–7) significantly (P < 0.05) reduced the expression of these genes.
caused an $82 \pm 8\%$ reduction in $\alpha$-SMA compared with vehicle ($P < 0.05$).

**Ang-(1–7) reduces hydroxyproline production by HSCs via the mas receptor**

Ang-(1–7) treatment also resulted in an overall decrease in collagen production, as reflected by a reduction in hydroxyproline levels in the supernatant compared with vehicle (Figure 6). The Ang-(1–7) receptor agonist AVE 0991 caused the greatest decrease in hydroxyproline levels ($14\%$ reduction; $P < 0.05$). Pre-treatment with the mas receptor antagonist A779 abolished the Ang-(1–7)-dependent decrease in hydroxyproline ($109 \pm 7\%$; $P < 0.05$).

**DISCUSSION**

Indirect evidence for a beneficial role of Ang-(1–7) in hepatic fibrosis has been provided by a previous study examining the effects of the mas receptor antagonist A779 [19]. Treatment with A779 worsened experimental liver injury with increases in TGF-\(\beta\)1 (transforming growth factor-\(\beta\)1) and hydroxyproline levels, suggesting that mas receptor stimulation plays a protective role in liver fibrosis [19]. Although mas is the putative receptor for Ang-(1–7), it has been suggested that other receptors may mediate the effects of Ang-(1–7) [14]. In the present study, we therefore provide the first direct evidence that Ang-(1–7) ameliorates hepatic fibrosis in an in vivo animal model. Furthermore, we demonstrate that plasma Ang-(1–7) levels are markedly elevated in human liver disease. We found that Ang-(1–7) levels were elevated in both groups of patients with hepatitis C, not just those with cirrhosis, whereas only cirrhotic patients had elevated AngII levels. Cirrhotic patients in the present study had advanced hepatitis C-related cirrhosis and were on the liver transplant waiting list, and represent a subgroup of cirrhotic subjects with advanced liver disease associated with portal hypertension and its associated haemodynamic changes. Thus our present results suggest that elevated levels of circulating Ang-(1–7) are related to the presence of hepatitis C-mediated liver injury and fibrosis, rather than the haemodynamic effects of portal hypertension on the RAS. This is in keeping with a study in a rat model of liver fibrosis which showed that chronically injured liver expresses ACE2 and this is primarily responsible for elevated serum Ang-(1–7) levels [18].

The Ang-(1–7)/AngII molar ratio in cirrhotic patients was significantly lower than that of non-cirrhotic and

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**Figure 3** *In vitro ACE autoradiography in the sham + saline, BDL + saline and BDL + Ang-(1–7) groups*

(A) Representative colour-gradient images from each group are shown. The areas of maximal radiodensity appear red, whereas the low radiodensity areas appear blue. (B) Quantification of ACE autoradiography [standardized relative absorbance (optical density)], showing that BDL resulted in a large increase in ACE protein expression, which was reduced to near sham levels by Ang-(1–7) treatment.
Figure 4  Gene expression of the key enzyme and receptor components of the RAS (ACE2, ACE, mas and AT₁R), together with markers of hepatic fibrosis (CTGF, collagen 1A1 and α-SMA) and angiogenesis (VEGF) in the sham + saline, BLD + saline and BDL + Ang-(1–7) groups

The treatment groups [BDL + Ang-(1–7) and BDL + saline] were normalized to the sham group, which is given an arbitrary value of 1 (n = 11 in each group).

control subjects. The likely explanation is that cirrhotic patients had elevated ACE levels and AngII concentrations due to the up-regulation of the vasoconstrictor arm of the RAS in response to portal hypertension and vasodilation [29]. The fact that, in cirrhotic patients, Ang-(1–7) concentrations were not increased in proportion to AngII makes it highly unlikely that serum Ang-(1–7) levels simply reflect the breakdown of circulating AngII. Indeed, the much lower Ang-(1–7)/AngII molar ratio in cirrhotic patients may reflect an increased ACE-mediated conversion of Ang-(1–7) into the inert peptide Ang-(1–5). These findings are in keeping with the hypothesis that relative angiotensin peptide levels are determined by the balance between ACE and ACE2 activity [15].

Infusion of Ang-(1–7) markedly attenuated hepatic fibrosis in the BDL rat, as assessed by blinded histological assessment using the METAVIR and ISHAK fibrosis scoring systems, and objective quantification of collagen content using a hydroxyproline colorimetric assay and computerized morphometric quantification of Picosirius Red staining. In addition, treatment with this peptide reduced HSC activation as shown by decreases in α-SMA gene expression and staining for α-SMA in areas of active fibrogenesis. Ang-(1–7) infusion also resulted in the down-regulation of key genes involved in hepatic fibrosis and angiogenesis, including collagen 1A1, CTGF and VEGF. Many of these effects are in direct opposition to those observed in the normal and fibrotic rat liver following AngII infusion [20,30]. Thus, in keeping with findings in cardiovascular and renal disease, in the liver Ang-(1–7) appears to be a potent inhibitor of experimental tissue fibrosis [8].

Our findings in HSCs provide a potential explanation for the antifibrotic effects of Ang-(1–7) in the BDL liver. Previous studies have shown that HSCs express AT_{1}Rs and that AngII up-regulates AT_{1}R and α-SMA gene
Ang-(1–7) induced a dose-dependent reduction in HSC α-SMA content

The authors found that Ang-(1–7) and AVE0991 treatment reduced the hydroxyproline content by 7 and 14% respectively. Pre-treatment with the mas receptor antagonist A779 abolished the Ang-(1–7)-mediated decrease in hydroxyproline. *P < 0.05 compared with AVE0991; †P < 0.05 compared with vehicle and 1 × 10⁻⁹ mol/l Ang-(1–7).

Infusion of Ang-(1–7) caused the down-regulation of the mas receptor and the ACE gene, but had no effect on ACE2 gene expression. This is particularly interesting given that Ang-(1–7) is primarily degraded by ACE into the inactive peptide fragment Ang-(1–5). Hence delivery of Ang-(1–7) appears to inhibit gene expression of the enzyme responsible for its degradation. This apparent regulatory interplay between RAS enzymes and their peptide substrates has also been shown for AngII, which reduces ACE2 gene expression but has no effect upon ACE [39]. It appears quite plausible therefore that AngII and Ang-(1–7) impart differential transcriptional regulation upon ACE2 and ACE respectively. ACEIs and ARBs have been shown to moderate hepatic fibrosis in several animal models. It is known that ACEIs block Ang-(1–7) breakdown, whereas ARBs increase ACE2 expression, and both class of drugs increase Ang-(1–7) concentrations [40]. These observations raise the possibility that, as in other organs, some of the beneficial effects of ACEIs and ARBs in experimental liver injury result from increases in Ang-(1–7) concentrations, rather than solely from a decrease in AngII formation or receptor binding [41–45].

VEGF is an important angiogenic factor and HSC mitogen that is elevated in hepatic fibrosis and hepatocellular carcinoma [46,47]. Although Ang-(1–7) has been shown to have anti-angiogenic and antimitogenic properties in other tissues [7,48] the effects of Ang-(1–7) on the hypoxia-responsive VEGF gene have not previously been described in the liver. The present study has demonstrated for the first time that the VEGF gene is down-regulated by Ang-(1–7), an effect which is directly opposite to that observed previously with AngII [49]. This finding is in line with a previous study that showed that mice lacking the ACE2 gene had up-regulated hypoxia-responsive genes in the heart [50], which may be due, at least in part, to decreased Ang-(1–7) formation. Likewise, ACE2 expression, leading to increased hydroxyproline production in these cells, an effect that is inhibited by AT1R blockade [31,32]. We were able to confirm that AT1R expression is increased during HSC activation; however, this is associated with a major up-regulation of mas, the putative Ang-(1–7) receptor, which mediates opposing actions to those of AT1R [13]. In keeping with these findings and its effects in the BDL liver, Ang-(1–7) caused a dose-dependent decrease in cellular α-SMA concentration, implying that it inhibits HSC activation. The non-peptide Ang-(1–7) mas receptor agonist AVE0991 at a dose of 1 × 10⁻⁶ mol/l produced the greatest decrease in α-SMA, perhaps reflecting its superior stability in culture. It also resulted in the largest decrease in collagen production as assessed using the hydroxyproline assay. Importantly, these effects were inhibited by pre-treatment with the mas receptor antagonist A779, suggesting that the antifibrotic effects of Ang-(1–7) are mediated via this receptor.

The functions and effects of ACE2 within the RAS are only now being realized [15,33]. This enzyme appears to play an important role in the response to cardiovascular [34,35] and renal [36] injury, and has also been identified as the receptor for the SARS (severe acute respiratory syndrome) coronavirus [37]. In agreement with our previous studies [17,18], hepatic ACE2 mRNA expression was significantly up-regulated in livers from cirrhotic patients with hepatitis C and in fibrotic rat liver, suggesting that this enzyme may play an important role in liver disease by increasing both Ang-(1–7) production and AngII degradation. Thus it appears that the ACE2-dependent arm of the RAS is up-regulated in response to hepatic injury and may help protect the liver from deleterious effects of locally generated AngII [16,38].
overexpression protects cultured cardiac fibroblasts from hypoxia-induced gene regulation [51]. Similarly, in the present study, the gene expression of the important growth factor CTGF (CCN-2) was significantly reduced by Ang-(1–7) treatment. CTGF has been implicated in the pathogenesis of fibrosis and, as with VEGF, may be increased by environmental factors such as local tissue hypoxia [52]. Thus the down-regulation of both CTGF and VEGF represent possible mechanisms by which Ang-(1–7) ameliorates hepatic fibrosis.

There is considerable evidence that AngII, the principal effector peptide of the vasoconstrictor arm of the RAS, has a key role in the initiation and perpetuation of inflammation and fibrosis in experimental liver injury. Most of the research to date investigating the role of the RAS in liver disease has concentrated on manipulating this ACE/AngII/AT1R axis of the RAS by blocking either ACE or AT1R. The present study provides strong evidence that the alternative ACE2-dependent arm of the RAS is activated by liver injury in humans and that its major peptide product, Ang-(1–7), is increased. This peptide decreases hepatic fibrosis in the BDL model and reduces collagen secretion by cultured rat HSCs through mas-dependent pathways. These findings suggest that agents capable of modulating the ACE2/Ang-(1–7)/mas receptor axis of the RAS have therapeutic potential in chronic liver disease.

FUNDING

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REFERENCES

Angiotensin-(1–7), an alternative metabolite of the renin–angiotensin system, is up-regulated in human liver disease and has antifibrotic activity in the bile-duct-ligated rat

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Table S1 Probes and primers for the Q-PCR analyses

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<th>Gene</th>
<th>Primer/F, forward primer</th>
<th>Primer/R, reverse primer</th>
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<td>ACE2</td>
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<td>Primer/R 5'-CTGAGAGTTCCACCTGC-3'</td>
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<td>ACE</td>
<td>Probe 5'-CAACAACACTGGCCTGCTG-3'</td>
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<tr>
<td>mas</td>
<td>Probe 5'-GGGATCCCTCTCATGCTG-3'</td>
<td>Primer/F 5'-CATCTCCACCTCTGGGTT-3'</td>
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<tr>
<td>AT1R</td>
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