Urotensin II modulates hepatic fibrosis and portal hemodynamic alterations in rats

William Kemp,1,2 Andrew Kompa,1 Arintaya Phrommintikul,1 Chandana Herath,3 Jia Zhiyuan,3 Peter Angus,3 Catriona McLean,4 Stuart Roberts,2 and Henry Krum1

1Department of Epidemiology and Preventive Medicine, Monash University; Departments of 2Gastroenterology and Hepatology and 3Anatomical Pathology, Alfred Hospital; 4Department of Medicine, University of Melbourne, Austin Health, Melbourne, Australia

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Urotensin II (UII), a vasoactive peptide with structural similarity to somatostatin, is found in the circulation of a wide range of species including man (7). Although the role of UII in normal physiology is unclear, in systemic resistance vessels, it is the most potent vasoconstrictor known, being an order of magnitude more potent than endothelin 1 (1, 10). In addition to its vasoconstrictive action, UII has been shown to induce vasodilation in the mesenteric vasculature in rats and human pulmonary and abdominal resistance vessels (30). The mechanisms underlying the varying vasoactive actions of UII in different vascular beds are only partially understood. However, they appear to be mediated by signaling via a specific G protein-coupled receptor (GPR-14) (1, 19, 24).

UII has several other potentially important physiological and pathological actions. These include induction of vascular smooth muscle cell and endothelial cell proliferation (29), acceleration of macrophage foam cell formation (34), stimulation of cardiomyocyte hypertrophy (16), and positive inotropic effects (25, 26), and it also induces collagen production in cardiac fibroblasts (18, 32, 35) via upregulation of transforming growth factor-β (TGF-β) (8).

Chronic liver disease and portal hypertension are characterized by activation of the renin-angiotensin-aldosterone system in addition to elevated plasma levels of potent vasoactive peptides such as noradrenaline and endothelin (2, 14, 20–22). Vasoactive peptides have a range of important effects, which may contribute to the pathogenesis of chronic liver disease. These include modulation of systemic and renal vascular tone, increase of portal pressure by promoting contraction of activated hepatic stellate cells, and stimulation of hepatic collagen production. Recently, it has been shown that UII levels are also increased in cirrhosis (15, 17) and that there is a correlation between the degree of UII elevation and both the severity of underlying liver disease and degree of portal hypertension (17). Recent studies also indicate that portal pressure can be reduced acutely with the use of a UII receptor antagonist (31). However, whether the known vasoactive, promitogenic, and profibrogenic effects of UII contribute to the pathogenesis of portal hypertension and hepatic fibrosis is unknown.

In this study, we therefore sought to examine the effects of chronic elevation of circulating UII on portal hemodynamics and hepatic fibrosis in normal rats.

MATERIALS AND METHODS

Animals. Twenty male Sprague-Dawley rats (200–250 g) were randomized into three treatment groups (control, N = 7; low dose rat, 1 nmol·kg⁻¹·h⁻¹ UII, N = 8; high dose rat, 3 nmol·kg⁻¹·h⁻¹ UII, N = 5). All rats were implanted with an osmotic mini-pump (Alzet mini-osmotic pump, 0.25 μl/h, Durect, Cupertino, CA), which provided continuous infusion over 28 days. Controls received saline vehicle only. Rats were housed in cages containing 2–4 rats and maintained on standard rat chow diet with water ad libitum.

Animal experiments were conducted in accordance with and were approved by the Alfred Medical Research and Education Precinct (AMREP) Animal Ethics Committee and conformed to the requirements of the National Health and Medical Research Council: Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th Edition, 2004).

Rat surgery. Forty-eight hours before insertion, mini osmotic pumps were filled with rat UII (GSK, King of Prussia, PA) spiked with 10⁶ cycles/min of [¹²⁵I]-UII, and incubated at 37°C. Radioactivity of the pump was measured before pump insertion and after 28 days to accurately determine the dose of UII administered during the treatment period.

Anesthesia was achieved via intraperitoneal injection with a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg). The pump reservoir (200 μl) was inserted subcutaneously under the right shoulder, and the infusion catheter was secured into the right internal jugular vein. The pump reservoir was connected to 28-gauge catheters that were inserted into the femoral arteries and veins and connected to a peristaltic pump (model 104, Harvard Apparatus, Holliston, MA) that delivered UII at a constant rate. After 28 days, rats were sacrificed, and portal and hepatic hemodynamics were measured.

Uropeptin II infusions were associated with an increase in hepatic transcript for transforming growth factor-β (TGF-β) (8). Increased plasma UII levels have been observed. In this study, we therefore sought to examine the effects of chronic elevation of circulating UII on portal hemodynamics and hepatic fibrosis in normal rats.
jugal vein. The wound was closed, and postoperative pain was treated with a single subcutaneous administration of Temgesic (but- 
oprophen, 0.03 mg/kg).

Echochardiography and hemodynamic analysis. Transthoracic echocardiography was performed on all rats under light anesthesia with intraperitoneal ketamine and xylazine (40 mg/kg and 5 mg/kg, respectively) at baseline and at day 28. Two-dimensional and M-mode images at the midpapillary muscle level of the heart were obtained using a HP Sonos 5500 with a 12-MHz probe (Agilent Technologies, Palo Alto, CA). Left ventricular (LV) internal diameters in systole and diastole were measured offline, and the percentage of LV fractional 
shortening was determined (28).

Hemodynamic measurements were performed in anesthetized ani-

(mals (sodium pentobarbionate, 50 mg/kg) following 28 days of UII infu-

sion. A precalibrated saline-filled catheter attached to a pressure 

transducer (UII, model 1050) was inserted into the right carotid artery 

and advanced to the aortic arch for measurement of systemic arterial 

pressure. Traces were recorded on a MacLab/2e system (ADInstruments, 

Castle Hill, Australia) and analyzed using Chart v3.6.8 (ADInstruments) 
to determine mean arterial pressure (MAP) and heart rate (HR). A 

second catheter was inserted into the portal vein by direct puncture, 

and portal pressure was measured. Animals were then euthanized, and 
tissues were harvested and archived at −80°C until reverse transcrip-
tion.

Reverse transcription and real-time PCR. Messenger RNA (mRNA) 

was reverse transcribed to cDNA using Multi-Scribe Reverse Transcrip-
tase (Applied Biosystems, Foster City, CA), and cDNA aliquots 

(5 ng each) were amplified using sequence-specific primers (Gen-

eworks, Adelaide, SA, Australia) and a TaqMan fluorescent probe (Ap-

plied Biosystems) using an ABI Prism 7900HT Sequence Detection 

System (Applied Biosystems). 18S (Applied Biosystems) was used 
as the endogenous control. Each 10-μl PCR mixture contained 

0.05 μl of ATG, 0.5 μl primers (forward and reverse), 0.5 μl probe, 

and 1 μl of cDNA template. PCR was performed at 50°C for 2 min 

and 95°C for 10 min, followed by 40 amplification cycles with 1-min 

annealing/extension at 60°C and 15-s denaturation at 95°C. PCR 

reactions for each tissue sample were carried out in triplicate, and 

results were expressed as the ratio to 18S. Probes and primers are 

listed in Table 1.

Statistics. Statistical analysis was conducted using SAS version 8.2 

(SAS Institute, Cary, NC) and SPSS version 13.0 (SPSS, Chicago, 

IL). All data are expressed as means ± SE unless stated otherwise. 

Continuous data were analyzed using Student’s t-test or ANOVA 

where appropriate and validated by Wilcoxon Rank Sum/Mann-

Whitney U-test or Kruskal-Wallis Test. Proportions were compared 

with Chi-squared test where appropriate. A two-sided P value of 0.05 

was considered to be statistically significant.

RESULTS

All rats survived the surgery without complication and received 28 days of infusion. Among rats receiving UII, base-

line weights were slightly heavier in the high-dose group; however, all rats gained weight appropriately during the study 

(Table 2). Taking into account the difference in radioactivity 

decay, the average dose of UII administered in the low-

and high-dose groups was 1.23 nmol·Kg⁻¹·h⁻¹ and 3.38 

nmol·Kg⁻¹·h⁻¹, respectively.

Table 1. Probe/primer sequences

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Probe/Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S</td>
<td>Probe</td>
<td>VIC-AGT CCA CTT TAA AGT CTT - MGB-NFQ · MGB-NFQ</td>
</tr>
<tr>
<td></td>
<td>Forward</td>
<td>5’-TGG AGG CCC TGT ATT TGG AA-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-CCC TCC AAT GGA TCC TGG TT-3’</td>
</tr>
<tr>
<td>Collagen 1</td>
<td>Probe</td>
<td>FAM-CTG CCG CTT ATG TCC ACC GAG - MGB-NFQ</td>
</tr>
<tr>
<td></td>
<td>Forward</td>
<td>5’-TTC CCA GCA TGT CGG TAT CCA-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-TCT TGC AGT GAT AGG TGA TGT TCT G-3’</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Probe</td>
<td>FAM-CAC CGT CAA GAC CAT - MGB-NFQ</td>
</tr>
<tr>
<td></td>
<td>Forward</td>
<td>5’-CCA GCC GGC GGA CTC T-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-TTC GGT TGG AGC AGC TGC ATT-3’</td>
</tr>
<tr>
<td>cTGF</td>
<td>Probe</td>
<td>FAM-AGG CTT GCC AAA TAC CAG - MGB-NFQ</td>
</tr>
<tr>
<td></td>
<td>Forward</td>
<td>5’-GGG GCC AGT CCT TCC AA-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-CCA GCG CCC CAT CCA-3’</td>
</tr>
<tr>
<td>PDGF-β</td>
<td>Probe</td>
<td>FAM-AGG TGC TCC AGA TCT CGG GGA ACC TCT-MGB-7AMRA</td>
</tr>
<tr>
<td></td>
<td>Forward</td>
<td>5’-CTT CGT CAA GAC GGC TAC A-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-GGG ATT GGT GGC ATC GA-3’</td>
</tr>
</tbody>
</table>

TGF, transforming growth factor; PDGF, platelet-derived growth factor; cTGF, connective tissue growth factor.
Day 28 organ weights are expressed as a percentage of baseline total body weight (Table 2). There was no difference in heart or liver weights with UII infusion; however, UII produced a dose-dependent significant increase in splenic mass (0.24%, 0.27%, and 0.29% of body weight respectively, \( P < 0.01 \)). In addition, renal mass was significantly increased in the low-dose group (\( P < 0.05 \)) with a nonsignificant increase noted in the high-dose group compared with controls. Biochemical and hemodynamic data are shown in Table 3.

**Hemodynamic parameters.** A dose-dependent increase in portal pressure was observed with UII infusion (Fig. 1) with the portal venous pressure at day 28 being significantly higher in the high-dose group compared with controls (7.6 ± 0.7 vs. 5.8 ± 0.4 mmHg, \( P < 0.05 \)).

There was no difference in the systemic hemodynamic parameters of MAP or HR with infusion of UII. Consistent with this, the echocardiographic data showed no effect on ejection fraction and systolic function with UII infusion (data not shown). However, UII infusion induced dose-dependent cardiac diastolic dysfunction as demonstrated by an increase in the mitral valve inflow deceleration time (0.057 ± 0.003, 0.093 ± 0.005 and 0.12 ± 0.022 s, respectively, \( P < 0.005 \)). Inferior vena cava (IVC) pressures were elevated in the high-dose group compared with low-dose group (1.9 ± 0.73 and 0.09 ± 0.31 mmHg, \( P < 0.05 \)).

**Fibrosis.** There was an increase in hepatic collagen content in animals receiving UII compared with controls (Fig. 2) with the hydroxyproline content being significantly higher in the high-dose group compared with controls [589 ± 60 \( \mu \)g/g vs. 416 ± 35 \( \mu \)g/g, \( P < 0.05 \); median (25–75 IQ): 531 (491–714) and 411 (337–528), respectively]. There was no increase in hydroxyproline content of renal tissue between the three groups (555 ± 28.3, 579 ± 11.5, and 575 ± 20.8, for control, low dose, and high dose, respectively). However, a significant elevation (\( P = 0.03 \)) in hydroxyproline was observed in splenic tissue from the high-dose group (755 ± 72.2 \( \mu \)g/g) vs. low-dose group (537 ± 62.8 \( \mu \)g/g). This was not significant compared with control group (583 ± 94.2 \( \mu \)g/g).

Representative images of hepatic histology are shown in Fig. 3. Quantification of fibrosis using computer morphometrics demonstrated a dose-dependent increase in fibrous tissue around the portal tracts (Fig. 4), suggesting that the UII-mediated increase in hepatic collagen content was predominantly in the peripoortal area.

**Quantitative real-time PCR.** UII infusion produced a dose-dependent increase in hepatic TGF-\( \beta \)1 mRNA expression (Fig. 5A), which was significant at the high dose compared with controls (\( P < 0.05 \)). In addition, a dose-dependent increase in gene expression of platelet-derived growth factor (PDGF)-\( \beta \) was observed, which approached significance (\( P = 0.06 \)) (Fig. 5C). Furthermore, the high dose UII infusion produced a significant increase in connective tissue-derived growth factor expression compared with the low dose (Fig. 5B), and there was an

### Table 2. Baseline and day 28 weights

<table>
<thead>
<tr>
<th></th>
<th>Controls, ( N = 7 )</th>
<th>Low Dose, ( N = 8 )</th>
<th>High Dose, ( N = 5 )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight, g ± SE</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>239±6</td>
<td>216±5†</td>
<td>256±8*</td>
</tr>
<tr>
<td>Day 28</td>
<td>361±9‡</td>
<td>354±7‡</td>
<td>390±9‡</td>
</tr>
<tr>
<td><strong>Organ weights, % baseline ± SE</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td>0.47±0.02</td>
<td>0.47±0.01</td>
<td>0.47±0.02</td>
</tr>
<tr>
<td>Liver</td>
<td>5.5±0.36</td>
<td>5.65±0.15</td>
<td>5.36±0.19</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.24±0.01</td>
<td>0.27±0.01†</td>
<td>0.29±0.018</td>
</tr>
<tr>
<td>Total kidney</td>
<td>0.99±0.02</td>
<td>1.07±0.02†</td>
<td>1.01±0.017*</td>
</tr>
</tbody>
</table>

Values are means ± SE. Organ weights are standardized to baseline body weights. *\( P < 0.05 \) vs. low-dose group; †\( P < 0.05 \) vs. control group; ‡\( P < 0.05 \) vs. control.

### Table 3. Day 28 biochemistry and hemodynamic data

<table>
<thead>
<tr>
<th></th>
<th><strong>Control</strong></th>
<th><strong>Low Dose</strong></th>
<th><strong>High Dose</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total protein, g/l</strong></td>
<td>51.1±1.8</td>
<td>52.0±0.8</td>
<td>50.6±1.1</td>
</tr>
<tr>
<td><strong>Albumin, g/l</strong></td>
<td>12.9±0.6</td>
<td>12.4±0.3</td>
<td>12.2±0.2</td>
</tr>
<tr>
<td><strong>Bilirubin, ( \mu )mol/l</strong></td>
<td>&lt;2.0</td>
<td>&lt;2.0</td>
<td>&lt;2.0</td>
</tr>
<tr>
<td><strong>ALT, IU/l</strong></td>
<td>37.4±4.4</td>
<td>41.1±2.2</td>
<td>34.0±3.4</td>
</tr>
<tr>
<td><strong>gGT, IU/l</strong></td>
<td>&lt;5.0</td>
<td>&lt;5.0</td>
<td>&lt;5.0</td>
</tr>
<tr>
<td><strong>ALP, IU/l</strong></td>
<td>201.9±12.4</td>
<td>223±10.8</td>
<td>208±24.1</td>
</tr>
<tr>
<td><strong>MAP, mmHg</strong></td>
<td>117.1±8.8</td>
<td>111.9±3.1</td>
<td>123.2±7.9</td>
</tr>
<tr>
<td><strong>Heart rate, beats/min</strong></td>
<td>329.5±21.5</td>
<td>311.0±6.1</td>
<td>322.8±20.0</td>
</tr>
<tr>
<td><strong>Portal pressure, mmHg</strong></td>
<td>5.8±0.4</td>
<td>6.4±0.3</td>
<td>7.6±0.7*</td>
</tr>
</tbody>
</table>

Values are means ± SE. *\( P < 0.05 \) vs. control. ALT, alanine aminotransferase; gGT, \( \gamma \)-glutamyl transpeptidase; ALP, alkaline phosphatase; MAP, mean arterial pressure.
increase in collagen I mRNA expression, which reached significance in the low-dose group (Fig. 5D).

DISCUSSION

To our knowledge, this is the first study to investigate the impact of elevated circulating UII peptide levels on portal hemodynamics and liver fibrosis. Our major study findings include the demonstration that, in normal rats, 1) chronic elevation of UII produces a significant dose-dependent increase in portal pressure in the absence of systemic hemodynamic alternations and 2) that this is associated with an upregulation in the hepatic expression of transcripts of key profibrotic cytokines TGF-β and PDGF-β and with liver fibrosis as measured by hepatic hydroxyproline concentration. These novel data have potential pathophysiological relevance in the chronic liver disease population who are known to have increased peripheral blood UII levels that correlate with both the severity of liver disease and degree of portal hypertension.

A recent study showed that UII increased mRNA transcripts for procollagens α1 (I), α1 (III), and fibronectin in cardiac fibroblasts with a concomitant increase in collagen synthesis (32). Furthermore, we detected an increased ratio of collagen I:III in rat cardiac tissue in vivo after a 2-wk UII infusion (18). The present study is the first to examine whether UII has profibrotic effects in liver tissue similar to those observed in the heart. Our data demonstrate that the profibrotic actions of UII are not limited to the cardiovascular system and suggest that the elevation of UII levels in hepatic diseases could contribute to fibrosis progression. We found that chronic UII infusion resulted in a dose-dependent increase in TGF-β1 and collagen I mRNA expression and an increase in collagen synthesis in the liver indicated by an elevation in hydroxyproline content with accumulation of collagen in periportal areas. There was no significant difference in hydroxyproline between the low-dose and high-dose groups. This may be a reflection of the limited number of animals studied; however, it is also possible that a ceiling effect of UII on hepatic fibrosis was reached. This concept would be supported by the lack of spare receptor reserve theory postulated by Douglas et al. (9). The mechanism through which UII stimulates collagen synthesis is unclear although one potential mechanism is its ability to induce production of the fibrogenic cytokine TGF-β and increase type I and III collagen mRNA expression, an effect inhibited in cardiac tissues by UII receptor antagonists (5, 8).

Furthermore, the influence of UII on hepatic stellate cells remains to be elucidated.

There are a number of possible explanations for the dose-dependent effects of UII on portal pressure and splenic weight.
Within the rat vasculature, UII possesses potent vasoconstrictive activity in the thoracic aorta (10) and produces vasodilation in both mesenteric (4) and hind quarter vascular beds (12). Although mesenteric vasodilation has the ability to increase portal venous flow, this is unlikely alone to account for the observed increase in portal pressure given the low pressure of the normal portal system. Furthermore, recent evidence indicates that the mesenteric vasodilation observed with bolus UII infusion is not reproduced with a more sustained UII infusion (11). UII could also produce a dynamic increase in portal vascular resistance. The site of UII receptors is presently under investigation although there is evidence indicating that intrahepatic UII and UII receptor expression is concentrated on sinusoidal endothelial cells and Kupffer cells, and upregulation of these receptors was observed in a bile duct ligation model (31). An increase in sinusoidal resistance could therefore occur via a direct action of UII on the hepatic sinusoidal endothelium and hepatic stellate cells, resulting in an increase in intrahepatic resistance through myofibroblast contraction. UII can induce cellular contraction through a Rho-A/Rho-kinase-dependent mechanism (27, 33), and upregulation of Rho-A/Rho-kinase has been demonstrated to contribute to intrahepatic resistance in cirrhotic rats (36). Neither of these systems were the focus of the present study. Nevertheless, this hypothesis is supported by the increased portal pressure observed with acute administration of UII to cirrhotic rats and a reduction in portal pressure with the use of UII receptor antagonists (31). Our study provides the first evidence that chronic UII administration has portal pressure-elevating effects. In this instance, UII-mediated liver fibrosis might have increased hepatic resistance. However, the lack of discernable scarring or changes in the hepatic architecture on light microscopy makes this unlikely.

The most profound action of UII is as an arterial vasoconstrictor. Our finding that, despite this effect, MAP was not increased during chronic UII infusion was consistent with our previous rat studies, which demonstrated an absence of hemodynamic alterations with a 2-wk infusion of 300 pmol/l (18). Others have also demonstrated that hemodynamic changes observed with bolus dosing of UII are not necessarily sustained or fluctuate over time with more prolonged infusion dosing (13). Because of the previously noted association between UII and cardiac dysfunction (18, 32), we assessed cardiac function via transthoracic echocardiography. Although no change in systolic function was observed, there was evidence of diastolic dysfunction. This diastolic dysfunction is a possible explanation for the increase in IVC pressure that was noted in the high-dose group. Thus it is possible that the increase in portal pressure observed with UII infusion was secondary to its effects on cardiac function and IVC pressure rather than via modulation of intrahepatic resistance. However, it is noteworthy that hepatic histology revealed no features of hepatic congestion, while serum liver chemistry levels were unaltered in the UII groups compared with controls. Furthermore, fibrous tissue was located around the portal tracts rather than centrilobular fibrosis as might be expected in cardiac hepatopathy (23).

Several caveats to this study are needed. The number of rats used in this study was limited by the high cost of UII, and this increased the chances of statistical type two error. However, despite this, increases in TGF-β and collagen I mRNA transcripts and increased hepatic hydroxyproline content were found, which are of potential pathogenic importance. Second,
the clinical consequence of an elevation in portal pressure in rat cannot be directly extrapolated to humans given the species variability that has been demonstrated with regards to the action of UII. Finally, our study involved healthy normal rats, and therefore we can only speculate as to the pathophysiological role and effect(s) of an elevated UII level in the evolution and setting of established chronic liver disease.

In conclusion, the present study demonstrates that UII infusion over 4 wk into normal rats can increase portal pressure and induce an increase in hepatic fibrosis. These findings are potentially important for our understanding of the pathophysiology of portal hypertension given the increase in circulating UII levels in subjects with chronic liver disease. Further studies with specific UII antagonists will enable further delineation of the pathophysiology of UII and its implications in portal hypertension and chronic liver disease.

REFERENCES


