

Increased expression of urotensin II and GPR14 in patients with cirrhosis and portal hypertension

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Abstract. Urotensin II (UII) and its receptor (UT or GPR14) are involved in liver fibrosis and portal hypertension. Nevertheless, expression of the UII/UT system in the liver of patients with portal hypertension has not been elucidated. UII and UT gene expression were quantified in liver biopsy samples from patients with hepatitis-B-virus-associated cirrhosis and portal hypertension, and from normal controls by using quantitative real-time PCR. The liver distribution of UT was determined by means of immunohistochemistry and immunofluorescence. Western blot analysis was used to assess liver levels of UT. Simultaneously, we measured intraoperative free portal venous pressure (FPVP) and collected plasma for UII measurement by ELISA. UT expression at the mRNA and protein level was enhanced significantly in the liver of patients with cirrhosis and portal hypertension, compared with that in healthy controls. UT protein expression was concentrated mainly in the Kupffer cells and sinusoidal endothelial cells. In cirrhotic tissue, UII gene expression was increased 5-fold in comparison to that in normal liver tissue. Plasma UII level was higher in cirrhotic patients compared with controls and was correlated with FPVP ($r=0.807$; $P<0.001$) and UII mRNA in the liver ($r=0.802$; $P<0.001$). These findings suggest that the intrahepatic UII/UT system has an important pathophysiological role in cirrhosis and portal hypertension.

Introduction

Increased intrahepatic resistance is the initial event of increased portal pressure and development of portal hypertension in cirrhosis (1,2). Careful pathological studies have demonstrated that, in addition to the increased resistance caused by morphological changes in chronic liver diseases, a dynamic component of increased resistance is present that represents active contraction of contractile elements in the liver. These elements constrict in a reversible and graded manner in response to several agonists, thereby further increasing the intrahepatic resistance (3,4). Several vasoconstrictors, such as angiotensin II and endothelin (ET), have been shown to be involved in the increased vascular tone in cirrhosis (5-7). The most recent addition to this expanding family of vasomodulatory substances is urotensin II (UII), a somatostatin-like cyclic undecapeptide, which has been identified as the most potent mammalian vasoconstrictor known (8). In addition to its effects on vascular tone, UII possesses mitogenic and fibrogenic potential (9). *In vivo* and *in vitro* studies have suggested that UII has a role in myocardial and vascular remodeling associated with cardiovascular diseases (10,11).

Although originally isolated from the urophysis of teleost fish, UII peptides have been highly conserved through evolution from fish to humans (12). Conservation of this cyclic hexapeptide suggests a significant physiological function for UII. The identification in 1999 of human UII as the cognate ligand for the novel G-protein-coupled receptor GPR14 (renamed urotensin II receptor, UT) has been followed by rapid exploration of the UII/UT system in human diseases (8). The observation that basal hemodynamics and general vasopressor responsiveness are unaltered in UT knockout mice supports the suggestion that the receptor is functionally silent under basal conditions, but this may change in cardiovascular diseases (13). In addition to elevated levels in patients with heart failure, systemic hypertension, diabetes mellitus, and renal failure (14,15), increased plasma UII levels have been reported in patients with cirrhosis (16,17).

However, although plasma levels of UII are upregulated in cirrhosis, their physiological relevance is unclear. The expression of the UII/UT system in the liver of patients with

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Abbreviations: FPVP, free portal venous pressure; UII, urotensin II; UT, urotensin II receptor

Key words: liver cirrhosis, portal hypertension, urotensin II, GPR14

portal hypertension has not been elucidated. Accordingly, the present study sought to determine the expression of UII and UT in patients with cirrhosis and portal hypertension.

Materials and methods

Patients and tissue collection. With local research ethics committee approval and written informed consent, we recruited 23 patients (seven female) with portal hypertension and hepatitis-B-virus-associated cirrhosis. All 23 patients had histological or clinical evidence of cirrhosis and portal hypertension. They underwent transabdominal esophagogastric devascularization and splenectomy because of variceal bleeding (first to fourth event pre-admission). Control tissue was obtained from surplus liver biopsy material that was obtained for diagnostic purposes from 10 living donors before liver transplantation. All normal control subjects had normal liver function, were normotensive, and had no evidence of liver fibrosis (Table I). Patients with diabetes mellitus, chronic kidney disease (creatinine >177 mmol/l; blood urea nitrogen >9 mmol/l), systemic hypertension, and aortic valvular diseases were excluded. Biopsy specimens were divided into two portions. One portion was immediately snap frozen and stored in liquid nitrogen for subsequent RNA extraction. A second portion was fixed in neutral buffered formalin and embedded in paraffin for later immunohistochemical analysis. Blood (5 ml) was collected from each patient's forearm vein into tubes that contained EDTA and aprotinin (0.6 TIU/ml of blood) after induction of anesthesia and tracheal intubation. Blood samples were maintained on ice before centrifugation at $3,000 \times g$ for 20 min at 4°C, after which plasma was removed and stored at -80°C.

RNA extraction and complementary DNA synthesis. Frozen liver biopsy tissues, stored in liquid nitrogen, were homogenized. Total RNA was extracted from snap-frozen liver biopsy specimens by using the Trizol Reagent kit (Invitrogen Life Technologies, Carlsbad, CA, USA). The amount of RNA recovered was quantitated, and its quality was verified by ultraviolet absorbance spectrophotometry at 260 and 280 nm. The cDNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. Reactions were as follows: 25°C for 10 min, 37°C for 150 min, 85°C for 5 sec, and 4°C for 5 min before chilling on ice. The cDNA was stored at -20°C for future use.

Real-time PCR (SYBR Green). UII and UT gene expression were quantified by means of real-time PCR. The sequences of the primers used are shown in Table II. GAPDH, a house-keeping gene, was used as an internal control primer for target genes. All primers were obtained from Invitrogen (Beijing, China). The expression of candidate gene mRNA was measured by SYBR Green real-time PCR using an ABI 7500 instrument (Applied Biosystems), according to the manufacturer's instructions. The PCR was performed in 20 μ l buffer that contained 2 μ g cDNA, 1 μ l of each primer, and 10 μ l SYBR Green PCR Master Mix (Applied Biosystems). Comparative cycle threshold (Ct) calculations were all relative to the control group. GAPDH Ct values were subtracted from gene Ct values

to give a final Ct value. $\Delta\Delta$ Ct values were achieved by subtracting the average control Δ Ct value, and expression of UII and UT relative to the control was derived by using the equation $2^{-\Delta\Delta Ct}$ (18). The derived normalized values were the average of three runs.

Extraction and assay of plasma UII. Quantitative analysis of UII in plasma by ELISA was carried out using the procedure suggested in the Urotensin II (Human) ELISA kit (Phoenix Biotech Co., Ltd, Beijing, China). This showed cross-reactivity against ET-1 and angiotensin II, adrenomedullin, calcitonin gene-related peptide, pre-pro urotensin II and brain natriuretic peptide. All analysis was done in a single batch.

Reactivity of the antibodies was investigated with a competitive ELISA system, as described elsewhere (19), using biotinylated human UII and streptavidin conjugated with horseradish peroxidase (HRP) instead of HRP-labeled peptide.

In the ELISA, 50 μ l human UII positive control in buffer and 50 μ l of sample solution or UII standard solution in buffer were added to wells of a microtiter plate that was precoated with secondary antibody, and incubated at room temperature (20–23°C) for 2 h. Then, 25 μ l of rehydrated biotinylated UII in buffer (provided in the kit) was added to each well and incubated at room temperature (20–23°C) for 2 h. After washing with PBS, 100 μ l/well of HRP-streptavidin in buffer (provided in the kit) was added to the plate and incubated for 1 h. The plate was washed four times with PBS, and subsequently, 100 μ l TMB substrate solution was added to each well and incubated for 2 h in the dark. Reaction was stopped by adding 100 μ l of 2 N HCl, and absorbance was measured at 450 nm in a Microplate Reader Type 680 (Bio-Rad Laboratories, Richmond, CA, USA). The intensity of absorbance was inversely proportional to the concentration of UII in the sample.

Measurement of free portal vein pressure (FPVP). FPVP was measured directly during the operation. A 20-cm-long rigid silica gel catheter, 0.2 cm in diameter was inserted through the right gastroepiploic vein into the portal vein. The catheter was fixed and connected with a monitor by a transducer. The patients were placed in the supine position, and the mid-axillary line was defined as the baseline for FPVP measurement. If there was a difference of >1 cm H₂O between the readings, all the recordings were discarded and fresh readings were taken.

Immunohistochemistry. The paraffin-fixed samples were sliced into 4- μ m-thick sections and subjected to immunohistochemical staining using the EliVision™ Plus kit (Maxim Corp, Fuzhou, China), according to the manufacturer's protocol. Tissue sections were incubated with rabbit anti-UII receptor (human) antibody (1:200; LifeSpan BioSciences, Seattle, WA, USA) for 18 h in a moist chamber at 4°C and washed three times with PBS. An enhancer was added for 30 min, which was followed by three washes in PBS, addition of HRP-conjugated goat anti-rabbit for 20 min, and another three washes in PBS. Finally, immunoreactivity was visualized by reaction with diaminobenzidine (supplied ready-to-use, Maxim Corp., Fuzhou, China) for 10 min, and counterstained with Mayer's hematoxylin. For a negative control, the primary antibodies were replaced with PBS.



Charateristics	Patients (n=23)	Normal Controls (n=10)
Sex (M/F)	16/7	7/3
Age (y)	38±6.63	51±10.04
Cause of cirrhosis	hepatitis B	N
No. with ascites	4	N
Child-Pugh A/B/C	19/4/0	N
Mean arterial blood pressure (mmHg)	87.9±2.1	93.9±4.31
Bilirubin (mol/l)	30.81±9.10	6.30±2.98
Albumin (g/l)	35.3±3.59	41.8±1.93
Free portal pressure (cm H ₂ O)	29.31±2.40	N
Urotensin II (ng/ml)	2.70±0.45	1.57±0.50

Double staining. To indentify UT-positive cells, paraffin sections were double-stained using indirect immunofluorescence. UT/CD68 double staining was done with anti-UT (GPR14, sc-20940; Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by FITC-conjugated anti-rabbit IgG (Zhongshan, Beijing, China) and anti-CD68 (Zhongshan) followed by TRITC-conjugated anti-mouse IgG (Zhongshan). UT/CD31 double staining was done with anti-UT followed by FITC-conjugated goat anti-rabbit IgG and anti-CD31 (Zhongshan) and TRITC-conjugated goat anti-mouse IgG. All staining experiments were done with appropriate positive and negative controls. Sections were analyzed by fluorescence microscopy (Olympus BX51 microscope). Colocalization of membrane staining (CD68/UT and CD31/UT) was visualized with image analysis software (Image Pro Plus, version 5.0; Media Cybernetics, Silver Spring, MD, USA) by applying the Boolean 'AND' operator. This operator combined individual unmixing images into a new image that consisted solely of colocalized (double-stained) pixels, thus representing double-stained cells only (yellow).

Western blot analysis. Protein was extracted from liver samples (Protein Extractor IV; DBI, Shanghai, China), homogenized, and assayed using the bicinchoninic acid method (Pierce BCA Protein Assay kit; Thermo Fisher Scientific Inc., Rockford, IL, USA). The 40-μg protein samples were subjected to SDS-PAGE (80 V for 40 min on a 5% acrylamide stacking gel and 120 V for 70 min on a 10% running gel), and then transferred (390 MA for 70 min) to a nitrocellulose membrane (Hybond-C Extra Membrane 45, Amersham Biosciences, Uppsala, Sweden). The membranes were soaked in Tris-buffered saline (10 mmol/l Tris-HCl and 250 mol/l NaCl) that contained 5% non-fat powdered milk and 0.1% Tween-20 for 2 h to block nonspecific sites, and incubated with primary antibody, rabbit anti-human UT antibody (GPR14; Santa Cruz Biotechnology) overnight at 4°C in blocking solution. The resulting blots were washed and incubated with secondary antibody (HRP-linked goat anti-rabbit IgG) for 2 h at room temperature. Immunoreactivity was visualized using an enhanced chemiluminescence kit (Thermo Fisher Scientific). Films were scanned using the

Table II. Primer sequences for human UII, UT, and GADPH.

Gene	Primer sequence	Product size (bp)	Accession number
GADPH	F: 5' AGCCACATC GCTCAGACAC3' R: 5' GCCCAATAC GACCAAATCC3'	66	NM_002046.3
UII	F: 5'CGTCTATCTT GTGGCGATCA3' R: 5' CCCAGCATCT CTGGCAGTAT 3'	201	NM_006786.2
UT	F: 5' GTACGTCTAC GTGGTCAACCTG3' R: 5'CACGATGA AGGGGATGCT3'	67	NM_018949.1

UII, urotensin II; UT, urotensin II receptor.

Bio-Rad imaging system. Antibody dilutions were as follows: rabbit polyclonal anti-UT antibody (1:1,000; Santa Cruz Biotechnology) and HRP-linked goat anti-rabbit IgG (1:5,000; Immunology Consultants Laboratory Inc., Newberg, OR, USA). Proteins were detected via enhanced chemiluminescence (Thermo Fisher Scientific), and bands were quantified via scanning densitometry using the SCAN Control (Scanco-1.lnk) imaging system. Individual levels of GPR14 protein expression were normalized to β-actin.

Statistical analysis. The results are expressed as mean ±SD. Statistical analysis was performed using ANOVA and unpaired Student's t test as appropriate. P<0.05 was considered statistically significant. The relationship between liver serum UII and FPVP was analyzed using the Pearson correlation coefficient. Statistical analyses were performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA).

Results

Clinical data. Basic patient and control data are shown in Table I. All patients had evidence of cirrhosis by histopathological analysis. None of the patients had taken vasoactive drugs or somatostatin for 1 week before surgery. Blood pressure and portal pressure measured at the time of surgery are listed in Table I.

Liver UII and UT gene expression. Measurement of gene expression of UII with real-time PCR in RNA extracted from biopsy samples showed a 5-fold increase in UII mRNA compared with normal control samples (Fig. 1A). Similarly, measurement of UT expression showed an even more dramatic, ~13.6-fold increase in expression in patients with cirrhosis and portal hypertension compared with normal control tissue (Fig. 2B).

Plasma UII levels and correlation with FPVP and UII mRNA. Plasma UII levels were significantly higher in patients with cirrhosis than in healthy controls (P<0.001; Fig. 2A, Table I).

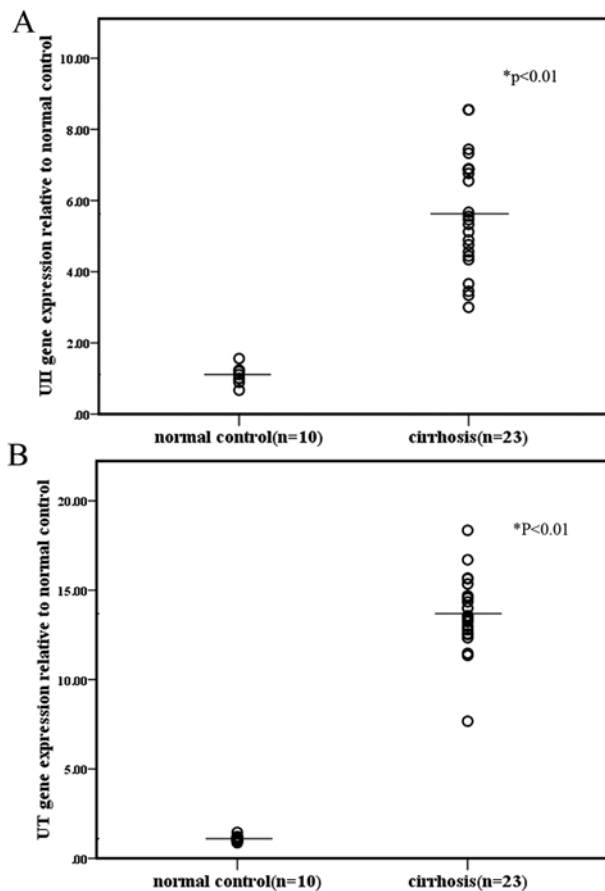


Figure 1. UII (A) and UT (B) mRNA in human liver tissue. mRNA was quantified in liver biopsy specimens from patients with cirrhosis and from normal living donors by real-time RT-PCR. Results are expressed as Ct for target gene standardized to Ct for the housekeeping gene GAPDH. Values expressed as mean (95% CI) relative to the controls, which were arbitrarily assigned a value of 1. * $P<0.01$ versus controls.

On the other hand, UII levels were correlated significantly with FPVP in patients with cirrhosis ($r=0.807$; $P<0.001$; Y (FPVP) = $18.954+0.616X$ (UII); Fig. 3). Plasma UII levels were correlated positively with UII mRNA in the liver ($r=0.802$; $P<0.001$; Fig. 2B).

Liver UT protein expression. Immunohistochemistry using anti-UT antibody showed little or no UT protein expression in the normal control liver. Occasionally, certain cell types, such as Kupffer cells and endothelial cells but not hepatocytes, expressed weak UT immunoreactivity (Fig. 4A and B). The liver of patients with cirrhosis and portal hypertension showed abundant UT protein expression, which was concentrated mainly in sinusoidal endothelial and Kupffer cells (Fig. 4C3 and C6). A 60-kDa band, indicative of UT expression, was observed for the liver (Fig. 5A). Western blot analysis demonstrated a significant increase in UT protein expression in the liver of patients with cirrhosis compared with the controls (Fig. 5B).

Discussion

Recently, several studies have shown that UII plasma levels are increased in patients with liver cirrhosis and portal hypertension (16,17). The UII plasma levels are correlated negatively

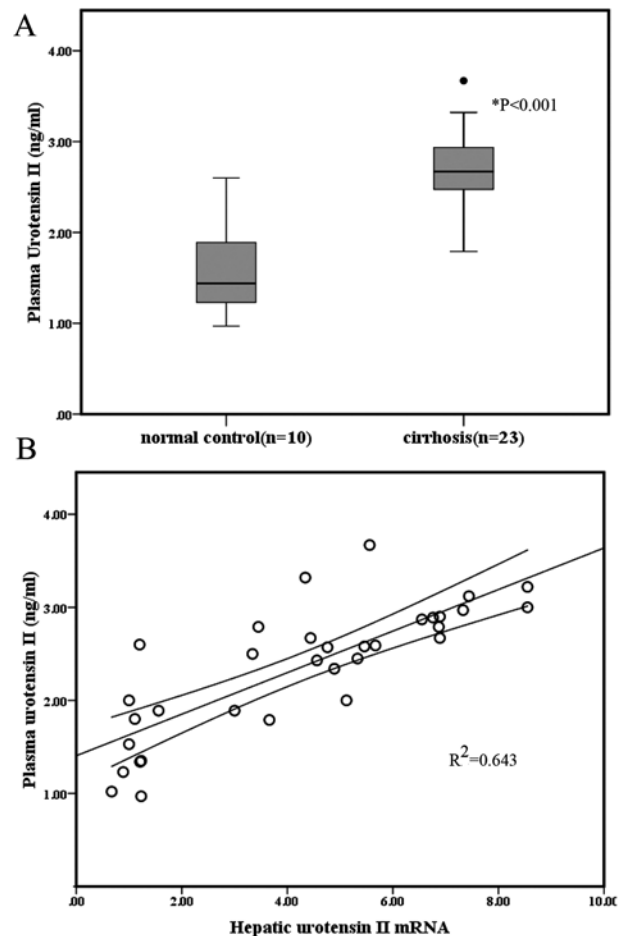


Figure 2. (A) UII plasma levels obtained from normal controls and 23 patients with cirrhosis and portal hypertension who were hemodynamically stable. Data are presented as the median. Plasma UII levels were significantly higher in patients with cirrhosis than in healthy controls ($P<0.001$). (B) Relationship of peripheral plasma UII levels and hepatic UII gene expression from 23 patients and 10 normal controls ($r=0.802$; $P<0.001$; $R^2=0.643$).

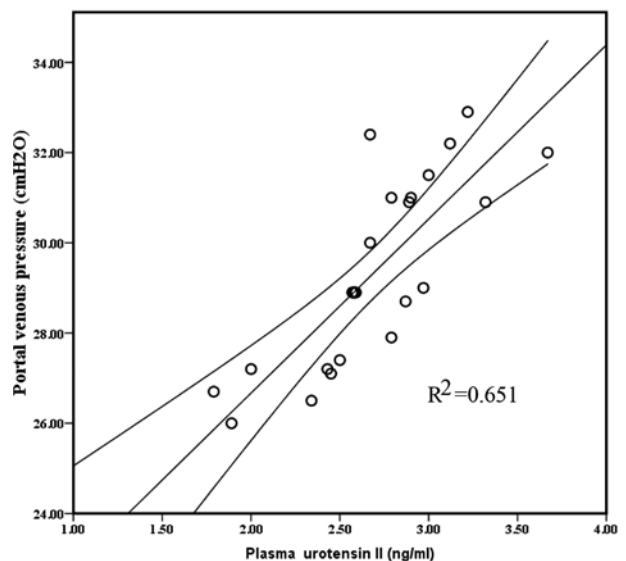


Figure 3. Correlation between UII plasma levels and FPVP obtained from 23 patients with cirrhosis ($r=0.807$; $P<0.01$; $R^2=0.651$; Pearson correlation test). Single-dimensional linear-regression is Y (FPVP) = $18.954+0.616X$ (UII).

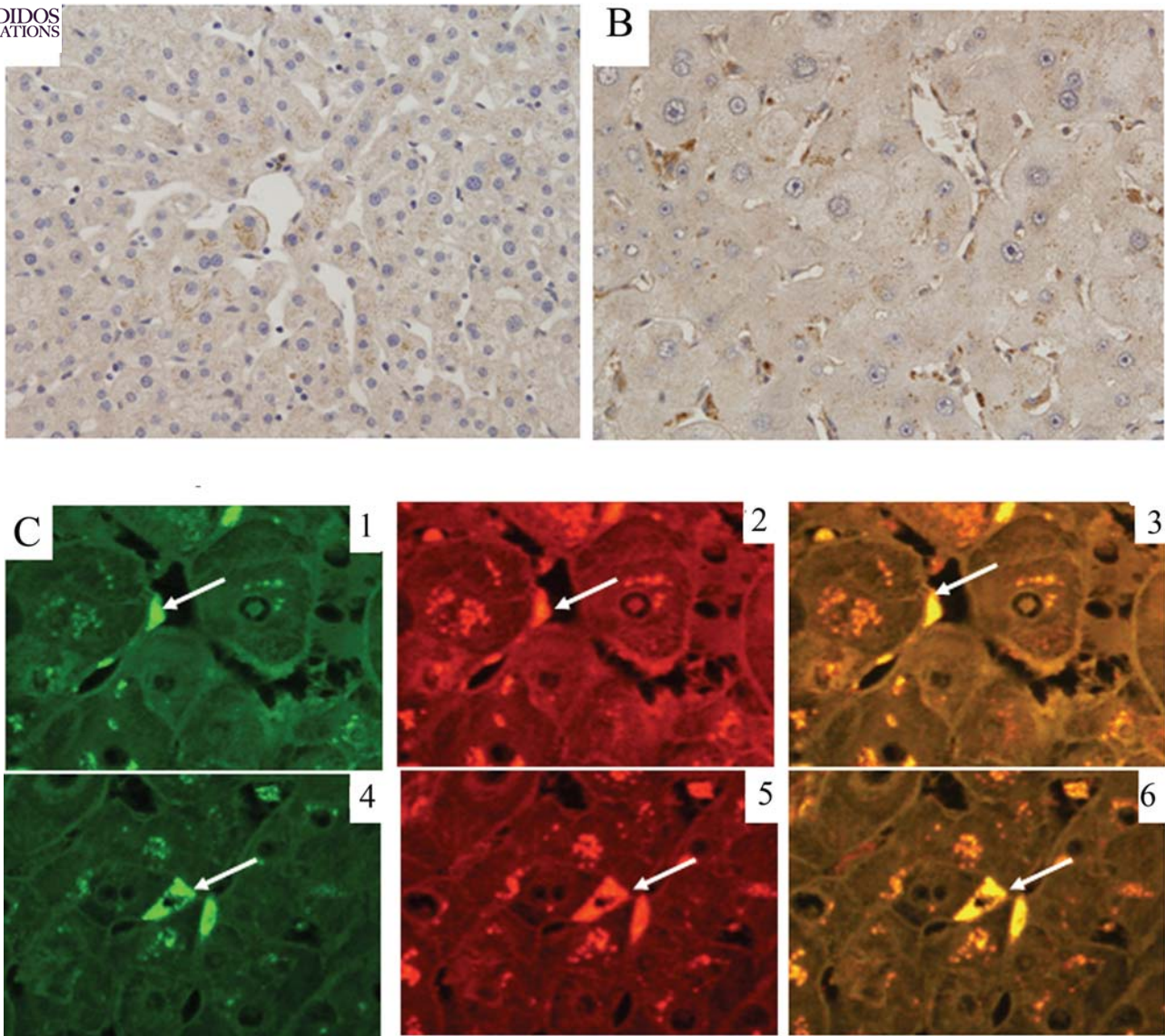


Figure 4. Immunohistochemical staining of UT protein in the liver of normal controls and patients with cirrhosis. In contrast to the little or no UT staining in the liver of normal controls (A), cirrhotic liver (B) displayed increased UT peptide expression. Co-localization experiments with UT (C1, arrow) and CD31 (C2, arrow) show that these cells were sinusoidal endothelial cells (C3, arrow). Co-localization experiments with UT (C4, arrow) and CD68 (C5, arrow) show that these cells were Kupffer cells (C6, arrow).

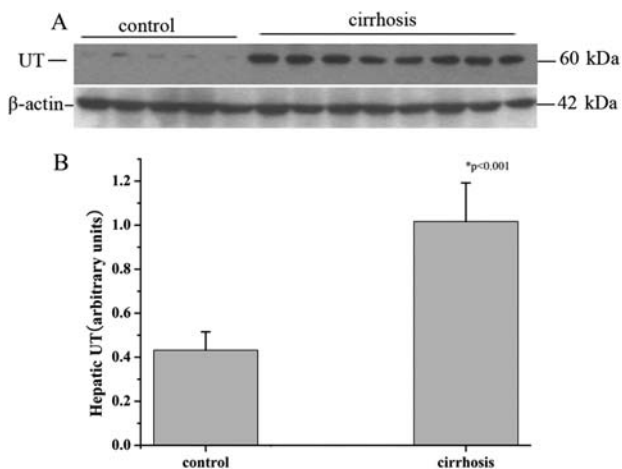


Figure 5. Protein expression of UT in the liver was determined by Western blotting in controls and patients with cirrhosis (A). Data were normalized to internal control (β -actin) and are expressed as the mean \pm SD * $p<0.001$ (cirrhosis vs. control) (B).

with mean arterial pressure and positively with portal pressure and severity of liver disease, as assessed using the Child-Pugh score (16,17). However, the site of increased production of UII in patients with chronic liver disease remains unclear (9). In addition, there are no published studies that have assessed liver UII/UT while simultaneously performing detailed intra-operative FPVP measurement and collecting plasma for UII determination. The simultaneous sampling and measurement of UT and UII as described in the present study are a significant advance, because we were able to describe in detail the physiology and molecular biology in live human subjects.

The present study showed dramatic overexpression of liver UII and its receptor in patients with cirrhosis and portal hypertension, with a 5-fold increase in ligand gene expression and an ~13.6-fold increase in receptor expression. In addition, immunohistochemical studies and Western blot analysis showed increased expression of UT in human cirrhotic liver, which is consistent with our previous study and that by

Tribeka *et al* in a rat model of cirrhosis (20,21). In apparent variance with our data, Leifeld *et al* have shown by immunohistochemistry that there was no significant difference in UII/UT between cirrhotic and control liver. There are likely to be many reasons for this disparity, including the diverse population group, rapid degradation of UII after its secretion, and marked differences in sensitivity and possibly specificity, between the UT assays (16,17,22), and its probable autocrine/paracrine mechanism of action (23,24). Other differences, besides etiology, are the absence of portal hypertension and plasma UII measurements in the study of Leifeld *et al*. Patients with cirrhosis and ascites (as confirmation of portal hypertension) have higher UII levels than those without ascites (17). This phenomenon seems not to be a feature of cirrhosis, but of portal hypertension. Portal hypertension seems to induce vascular and hepatic upregulation of UT and fibrosis, as shown recently by Kemp *et al* (25). Furthermore, a limitation of our study is that the patients with cirrhosis and portal hypertension are only a fraction of all patients with cirrhosis.

Although liver tissue expression of UII/UT mRNA in patients with cirrhosis and portal hypertension has not been examined previously, other studies have assessed elevated plasma UII levels in patients with chronic liver disease and those with portal hypertension (17). However, because of the diverse population group and potential confounding variables such as hepatorenal syndrome, and hemodynamic instability secondary to variceal bleeding, it is difficult to determine to what extent the observed increase in UII levels is attributable directly to the underlying liver disease (9). For instance, Heller *et al* (17) have demonstrated increased levels of UII in their patients with cirrhosis. The trans-hepatic gradient between the hepatic and portal vein suggests hepatic production of this peptide. However, alternative sites of UII production, such as the splanchnic circulation or portal venous system, cannot be excluded. More recently, UII concentrations have been determined in blood from the femoral and hepatic veins in patients with stable cirrhosis, who were undergoing assessment of portal hemodynamics, and have been found to be higher in the femoral than the hepatic vein. Although this implies systemic overproduction of UII rather than increased hepatic output (9,16), the data do not exclude the possibility of reduced hepatic clearance of UII. To address the issue of UII in the pathogenesis of portal hypertension, our group determined UII levels in patients with cirrhosis without hepatorenal syndrome, systemic hypertension, diabetes and hemodynamic instability secondary to variceal bleeding. UII levels in patients with heart failure, systemic hypertension, diabetes mellitus and renal failure are elevated (14,15), therefore, patients with diabetes mellitus, chronic kidney disease, systemic hypertension, and aortic valvular disease were excluded. UII circulates in the human plasma and its concentration is measured by ELISA using specific antibodies. Here, consistent with the study of Heller *et al* (17), we found significantly higher UII levels in patients with cirrhosis than in healthy controls, and UII levels correlated positively with the degree of FPVP. Furthermore, the enhanced expression of hepatic UII mRNA was found to be associated more closely with circulating UII levels. Together, these findings indicate up-regulation of UII expression by the liver, which leads to the possible increased release of UII into the circulation, in patients with cirrhosis and portal hypertension.

Although recognized for its potent vasoconstrictive actions, the effects of UII, like ET, are highly contextual, with responses dependent on vessel size, anatomical location, and species studied (26). For instance, although UII is a potent vasoconstrictor in large vessels of rodents and non-human primates, it also causes vasodilation of human pulmonary vessels (27,28). In humans, iontophoresed topical UII causes vasodilation in healthy controls but vasoconstriction in subjects with chronic heart failure (29) and hypertension (30). The difference is attributed to impaired endothelial function in patients, in whom endothelial cells normally produce nitric oxide and prostacyclin, which contribute to reduced vascular tone. In portal hypertension, however, NO is not produced in the presinusoidal system and its production is disturbed or substantially decreased in the sinusoidal/post sinusoidal area (2,31). In cirrhosis, increased intrahepatic vascular resistance is thought to be mainly in the hepatic sinusoids (32). In the present study, the expression of liver UT was upregulated in cirrhosis. Our immunohistochemical studies showed localization of UT in sinusoidal lining cells. This raises the possibility that UII has pathophysiological relevance in the portal hypertensive population through its vasoactive properties in hepatic sinusoids.

To date, most studies that have examined UII and UT have focused on the cardiovascular system, in which it is viewed as a potential target for therapeutic modulation (33). Cardiac remodeling after myocardial infarction, like liver sinusoid remodeling in liver cirrhosis, is characterized by excessive deposition of extracellular matrix (ECM) (34). Consistent with this pathogenetic role, *in vitro* studies of cultured fibroblasts have shown that UII induces the expression of fibronectin and collagen, to an extent similar to the profibrotic peptide, angiotensin II (35). Furthermore, induction of UT overexpression leads to augmented collagenous matrix synthesis (35). We have investigated the ability of UT receptor antagonist SB-710411 to arrest fibrosis in a rat model of liver cirrhosis (20). Together, the studies suggest that dramatic upregulation of UII and its receptor in liver cirrhosis found in the present study may contribute directly to the pathogenesis of the matrix expansion in patients with cirrhosis.

At a cellular level, no study has examined the effects of UII on hepatic stellate cells (HSCs), which have contractile and fibrogenic properties, and are located in the persinusoidal space of Disse, which is the principal site of ECM production (36). Several vasoconstrictors are involved in HSC activation, proliferation and constriction (6,37,38). UII is a potent vasoconstrictor peptide and its potency of vasoconstriction is one order of magnitude greater than that of ET-1 (39). UII induces proliferation of cultured fibroblasts and contraction of smooth muscle cells (40). It is speculated therefore, that UII is a stimulating factor for proliferation and contraction of HSCs. However, although the present study focused on biopsy specimens from patients with cirrhosis and portal hypertension, it is possible that the overexpression of UII in liver may not be of pathogenic significance. Additional studies to investigate the effect of UII on HSCs could help in establishing the role of UII in patients with cirrhosis and portal hypertension.

In summary, expression of UII and its receptor was dramatically increased in patients with cirrhosis and portal hypertension. Although the mechanism of action of UII



Further investigation, in the context of its known bio-effects, these findings suggest that this newly described vasoactive peptide system has a role in the pathogenesis of portal hypertension. Inhibition of this system could affect the progression of portal hypertension and have great clinical significance.

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