Eicosanoid Excretion in Hepatic Cirrhosis
Predominance of 20-HETE

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Abstract
The cytochrome P450 system transforms AA to hydroxy-eicosatetraenoic acid (HETE) metabolites that are vasoactive and affect transport in several nephron segments. A principal product of this system, 20-HETE, participates in key mechanisms that regulate the renal circulation and extracellular fluid volume. We hypothesized that excess production of 20-HETE, which constricts the renal vasculature, contributes to the renal functional disturbances in patients with hepatic cirrhosis, particularly the depression of renal hemodynamics. The development of a precise and sensitive gas chromatographic/mass spectrometric method makes it possible to measure 20-HETE and the subterminal HETEs (16-, 17-, 18-, and 19-HETEs) in biological fluids. As 20-HETE was excreted as the glucuronide conjugate, measurement of 20-HETE required treatment of urine with glucuronidase. We measured HETEs in the urine of patients with cirrhosis, and compared these values to those of normal subjects. Urinary excretion rate of 20-HETE was highest in patients with ascites; 12.5 ± 3.2 ng/min vs. 5.0 ± 1.5 and 1.6 ± 0.2 ng/min in cirrhotic patients without ascites and in normal subjects, respectively. Excretion of 16-, 17-, and 18-HETEs was not increased. In patients with cirrhosis, the excretory rate of 20-HETE was several-fold higher than those of prostaglandins and thromboxane, whereas in normal subjects 20-HETE and prostaglandins were excreted at similar rates. Of the eicosanoids, only increased excretion of 20-HETE in subjects with cirrhosis was correlated (r = −0.61; P < 0.01) with reduction of renal plasma flow (RPF). (J. Clin. Invest. 1997. 100:1264–1270.) Key words: hepatorenal syndrome • 16-, 17-, 18-HETEs • arachidonate metabolites • cytochrome P450 • renal eicosanoids • thromboxane

Introduction
During the course of cirrhosis, renal function progressively deteriorates without evidence of organic nephropathy (1). Functional renal impairment in compensated cirrhosis is subclinical, the alterations being a reduction of renal blood flow (RBF), (2, 3, 4), in particular the cortical component (5, 6), usually without depression of GFR (3, 4), a blunted response to a sodium load (7), and increased proximal tubular sodium reabsorption (8). When ascites develops, sodium and water excretion are demonstrably impaired, and RBF is decreased further (3, 4), effects that may be associated with reduced GFR. Subjects with cirrhosis and ascites show activation of the principal antidiuretic-antinatriuretic neurohumoral systems, the renin-angiotensin-adrenergic-aldosterone ADH axis (3, 9, 10), Renal vasodilator-diuretic systems, such as prostaglandins (3, 11–13), kallikrein-kinin (14, 15), and nitric oxide (16), are also activated in cirrhotic subjects with ascites. A vascular mechanism involving prostaglandins E2 (PGE2) and I2 (PGI2) (3, 12, 13) contributes to maintenance of RBF and GFR in patients with far advanced cirrhosis and ascites. The importance of a prostaglandin-dependent mechanism in cirrhosis with ascites is evident in the rapid decline in renal function produced by inhibition of cyclooxygenase with aspirin-like drugs (17). Thromboxane A2 (TxA2), on the other hand, may contribute to deterioration in renal function (12, 18).

Metabolites of AA that are generated by the cytochrome P450 (CYP) system have also been identified as major products of AA metabolism in the kidney (19). One of these products, 20-hydroxyeicosatetraenoic acid (20-HETE), that is produced in large quantities by the kidney, is vasoactive (20–23), and affects renal transport mechanisms (24, 25). 20-HETE is a principal product of AA metabolism in the proximal tubules (26), medullary thick ascending limb (mTAL) (27), and preglomerular microvessels (28). 20-HETE is a major component in several key mechanisms that regulate the renal circulation and salt and water excretion, and that are affected in cirrhotic subjects. 20-HETE has been shown to: (a) modulate the Na+–K+–Cl– cotransporter in the mTAL (24); (b) participate in tubuloglomerular feedback (29), and, (c) contribute to blood pressure elevation when produced in excess as in the spontaneously hypertensive rat (30). In terms of the genesis of renal dysfunction in association with hepatic cirrhosis, 20-HETE is produced in large amounts by preglomerular arterioles at which site it acts in the autoregulatory mechanism to constrict renal arterioles in response to increased perfusion pressure (28). 20-HETE has been identified in the human kidney, and is excreted conjugated to glucuronide (31); treatment with glucuronidase is required for measurement of the free form of urinary 20-HETE.

We have recently demonstrated that 20-HETE as well as 19-HETE and the subterminal HETEs, 16-, 17-, and 18-, are released selectively from the rabbit kidney in response to angiotensin II (AII); AVP and bradykinin were without effect.

1. Abbreviations used in this paper: 20-HETE, 20-hydroxyeicosatetraenoic acid; CYP, cytochrome P450; GC/MS, gas chromatographic/mass spectrometric; mTAL, medullary thick ascending limb; PAH, p-aminohippurate; PRA, plasma renin activity; RBF, renal blood flow; RPF, renal plasma flow.

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(32). HETEs effluxed primarily into the urine, venous efflux being a small component of the total (33). The subterminal HETEs were shown to be vasoactive, and to affect renal ion transport (32). This was the first report that an intact organ responded to hormonal stimulation by releasing HETEs.

The simultaneous measurement of 20-HETE and the subterminal HETEs (16-, 17-, 18-, and 19-) in biological fluids is now possible because of the development of a precise and sensitive gas chromatographic/mass spectrometric (GC/MS) method that combines the specificity of capillary column gas chromatography and the sensitivity of electron capture ionization mass spectrometry (32). We have applied this method to measuring HETEs in the urine of normal subjects and those with cirrhosis of varying severity. We designed this study to define a possible role of 20-HETE and related CYP-derived HETEs in the evolution of renal functional deterioration in patients with cirrhosis and ascites. Cirrhotic subjects with overt renal failure, as defined by serum creatinine levels > 2 mg/dl, were excluded because the focus of the study was renal dysfunction secondary to hepatic cirrhosis. Cirrhotic subjects similar to those in the present study have been reported to show either normal or slightly reduced GFRs associated with modest reductions in RBF (3, 4). In view of the considerable capacity to produce 20-HETE by that section of the renal vasculature (the preglomerular microvessels) primarily responsible for affecting changes in renal vascular resistance (28) as well as the vasoconstrictor potency of 20-HETE (23), we hypothesize that renal 20-HETE levels are increased in subjects with cirrhosis. This increase measured as urinary excretion of 20-HETE was correlated with the progression of liver failure and associated renal dysfunction. Of the HETEs, urinary excretion of 20-HETE exceeded by threefold the excretion of thromboxane, the predominant urinary cyclooxygenase product in cirrhosis with ascites.

Methods

Study population. 16 cirrhotic patients (8 ascites, 8 nonascites; 4 females, and 12 males; mean age 51±11 yr) were included in this study. The diagnosis of cirrhosis was histological in all cases; the etiology was alcoholic in 10 and posthepatic in 6. Among cirrhotic patients, eight were without ascites and eight had ascites with clinical and sonographic evidence of ascites. According to Child-Pugh criteria (34), five patients were grade A, five patients were grade B, and six were grade C. All patients had endoscopically demonstrated esophageal varices. Cirrhotic subjects were excluded if they had hyperglycemia (serum glucose > 120 mg/dl), renal failure (serum creatinine > 2 mg/dl), hyponatremia (serum sodium < 125 mEq/liter), hypokalemia (serum potassium < 3.0 mEq/liter), hyperkalemia (serum potassium > 5.0 mEq/liter), hypertension, or heart failure. At the time of the study, patients were stable; those with alcoholic liver disease had been abstinent for at least 2 mo before the study, and gastrointestinal hemorrhage had not occurred in the month before the study. Diuretics were discontinued 10 d before the study. The control population included five normal male subjects and three female (mean age 45±8 yr). History, physical examination, electrocardiogram, and routine chemical analyses excluded cardiovascular, renal, and hepatic dysfunction. None of the control subjects were on medication. The study was designed and performed according to the principles of the Declaration of Helsinki. The nature of the study was fully explained to the patients, and verbal informed consent was obtained in all patients.

Study design. Patients and controls were maintained for at least 5 d before the study on a normocaloric diet with a sodium intake of 80 mmol/d; 24-h urinary sodium excretion was used to control the diet. Water intake was restricted to 1 liter per day. For the 24 h before and throughout the study, beverages containing methylxanthines were excluded. At 9:00 pm of the day before the study, 600 mg of lithium carbonate was administered per osmole. On the following morning after an overnight fast, a urine collection period was started at 6:00 am and an intravenous priming dose of p-aminohippurate (PAH) (8 mg/kg) was given, followed by constant infusion of 5% dextrose/water at 1.5 ml/min for at least 5 h. The amount of PAH infused was calculated to maintain a serum PAH concentration of ~ 1.5 mg/dl. Blood samples were taken at the beginning, at midpoint, and at the end of the urine collection period (which lasted at least 7 h). Urine was collected by spontaneous voiding. To maintain an adequate diuresis, tap water 100–200 ml/hr was given. Patients remained supine throughout the study. Blood samples at the beginning and at the end of the urine collection period were analyzed for sodium (Na), lithium (Li), creatinine (Cr), and PAH concentrations. Blood samples at the midpoint of the clearance period were analyzed for plasma renin activity (PRA). Urine samples were analyzed for Na, Li, Cr, and PAH concentrations. Samples were also immediately frozen and kept at ~70°C for assay of PGE2, 6ketoPGF1α, TXβ2, and CYP-dependent HETE.

Analytic procedures. Na and Li were measured by flame photometry (Instrumentation Laboratory, Inc., Lexington, MA). Cr and PAH were determined colorimetrically. PRA was determined by RIA of angiotensin I (Renin Maia Kit; Bio/Data Corp., Hatboro, PA).

CYP-dependent HETEs. 25 ng of 19-HETE-d5 was added as an internal standard to 5-ml urine samples. Samples were allowed to equilibrate for 20 min at room temperature, and then incubated for 2 h at 37°C with 0.5 mg of β-glucuronidase (in 0.075 M potassium phosphate buffer, pH 6.8). Incubation with β-glucuronidase has been shown to increase urinary levels of 20-HETE ~ 20-fold (31), a result confirmed by preliminary experiments performed in our laboratory (see Fig. 1 B) After adjusting the pH to 3.0, each urine sample was applied to a solid-phase extraction column (Tox Elut; Varian Inc., Harbor City, California), allowed to sit for 15 min, and then eluted with 15 ml of dichloromethane. After evaporation to dryness, the residue was dissolved in methanol (20 μl), and purified by reverse-phase HPLC on a Ultrasphere ODS (250 × 4.6 mm, 5 nm; Beckman Instruments Inc., Fullerton, CA) column, with a linear gradient running from acetoni-trile:water:acetic acid 62.5:37.5:0.5 to acetonitrile 100% at a flow rate of 1 ml/min for 20 min. Fractions corresponding to elution of 16-, 17-, 18-, 19-, and 20-HETE standards and 19-HETE-d3 (8 and 9 min), were collected, dried, and dissolved in acetonitrile (100 μl). For GC/MS analysis, samples were converted to pentafluorobenzyl ester trimethylsilyl ethers by the addition of 30 μl of α-bromo-2,3,4,5,6-pentafluoro-toluene and 30 μl of N,N-disopropylethylamine for 30 min. After evaporation to dryness under nitrogen, trimethylsilyl ethers of free hydroxyl groups were prepared by reaction with 80 μl of bis(trimethylsilyl)-trifluoroacetamide and 20 μl of pyridine for 30 min. Samples were dissolved in isocetane, and 1-μl aliquots were analyzed by GC/MS. Gas chromatography (HPS890) was performed on a DB-1 column (15 m; 0.25-mm inner diameter, 0.25-μm film thickness; J & W Sci., Folsom, CA) using a temperature program ranging from 150–300°C at a rate of 1°C/min. The mass spectrometer (5989A; Hewlett-Packard Co., Palo Alto, CA) was operated in the negative ion chemical ionization mode (electron capture) with methane as the reagent gas at a service pressure of 1.6 Torr. The CYP-HETEs were identified by comparison of gas chromatographic retention times with authentic standards of 16- to 20-HETEs and quantitated by calculating the ratio of relative abundance with 19-HETE-d5.

Prostaglandins. PGE2, 6ketoPGF1α, and TXβ2 were measured in unextracted urine with the use of second-antibody, solid-phase, ELISA (35, 36). Purification by solid-phase column extraction, performed as previously described, did not improve the quality of the assay. Microtiter plates (immunoplate II; Nunc Inc., Naperville, IL) were coated with goat anti-rabbit IgG (200 μl per well, Boehringer Mannheim Biochemicals, Indianapolis, IN) at a concentration of 10 μg/ml and incubated at room temperature for 3 h. The plates were washed, and the plates were blocked with 1% BSA. The standard or sample was added, followed by incubation for 2 h at room temperature. The plates were washed, and the bound 6ketoPGF1α and PGE2 were detected using a rabbit antibody to 6ketoPGF1α and goat anti-rabbit IgG. The bound antibodies were detected with horse radish peroxidase-labeled rabbit antibody to goat antibody and o-phenylenediamine. 3,3′-diaminobenzidine tetrahydrochloride was added as a chromogen.
Table I. Renal Hemodynamic and Functional Parameters In Control and Cirrhotic Subjects

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control subjects</th>
<th>Without ascites</th>
<th>With ascites</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP (mmHg)</td>
<td>98±3</td>
<td>96±2</td>
<td>95±3</td>
</tr>
<tr>
<td>Plasma sodium (mEq/liter)</td>
<td>141±2</td>
<td>140±3</td>
<td>138±3</td>
</tr>
<tr>
<td>Sodium excretion (μEq/min)</td>
<td>156±13</td>
<td>161±23</td>
<td>114±13*</td>
</tr>
<tr>
<td>GFR (ml/min)</td>
<td>104±17</td>
<td>118±11</td>
<td>91±4</td>
</tr>
<tr>
<td>RPF (ml/min)</td>
<td>614±50</td>
<td>513±39</td>
<td>419±37*</td>
</tr>
<tr>
<td>Sodium clearance (ml/min)</td>
<td>1.25±0.12</td>
<td>1.55±0.34</td>
<td>0.83±0.12*</td>
</tr>
<tr>
<td>Lithium clearance (ml/min)</td>
<td>32.2±3.5</td>
<td>27.4±3.7</td>
<td>22.5±3.2*</td>
</tr>
<tr>
<td>PRA (ng/ml/h)</td>
<td>1.7±0.3</td>
<td>1.0±0.3</td>
<td>2.6±0.8</td>
</tr>
</tbody>
</table>

MAP, mean arterial pressure; RPF, renal plasma flow; PRA, plasma renin activity. *P < 0.05 vs controls.

Results

The hemodynamic and renal functional indices in patients with cirrhosis with and without ascites and in control subjects are shown in Table I. Blood pressure did not differ among the three groups as it was recorded in the supine position. In cirrhotic subjects with ascites, RPF was reduced and GFR was depressed (the latter not significantly) when associated with decreased lithium clearance, an index of proximal tubular re-absorption (37), and reduced urinary sodium excretion and clearance; PRA was marginally elevated.

Separation of CYP-HETE standards is shown in Fig. 1A. CYP-dependent HETEs were virtually undetectable in urine before incubation with β-glucuronidase (Fig. 1B). When urine from control and cirrhotic subjects was preincubated with β-glucuronidase, four CYP-derived HETEs (16-, 17-, 18-, and 20-HETE) were readily demonstrated (Fig. 1B). Two unidentified peaks migrating between 19- and 20-HETEs did not correspond to any known HETE standard. Lipoxygenase-derived HETEs such as 12- and 15-HETEs were separated from CYP-related HETEs during the extensive purification with HPLC. Any unseparated lipoxygenase-derived HETEs would have eluted at 7.4 min (Fig. 1). The two compounds, which were not characterized structurally, have to be considered as impurities,
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and were most evident in urine from cirrhotic subjects. 19-HETE could not be demonstrated in the urine of either control or cirrhotic subjects. Fig. 2 shows the difference in gas chromatographic analysis of urine from a control and a cirrhotic subject.

Urinary excretion of 20-HETE was significantly increased in cirrhotic patients, the largest increase occurring in those with ascites, whereas excretion rates of 16-, 17- and 18-HETE were not increased significantly in cirrhotic patients when compared to control subjects (Fig. 3). Renal plasma flow (RPF), which was significantly decreased only in cirrhotic subjects with ascites (Table I), correlated negatively with 20-HETE excretion (r = −0.61; P < 0.01) (Fig. 4), but did not correlate with excretion of 16-, 17- and 18-HETEs. 3 of the 16 subjects with cirrhosis, and 2 of the 8 control subjects were not included in Fig. 4 because renal clearances could not be ob-

Figure 2. Gas chromatographic/mass spectrometric analysis of 5-ml urine samples from a normal subject (left) and from a cirrhotic patient with ascites (right), preincubated with β-glucuronidase. Ions m/z 391 (top chromatograms) and m/z 394 (bottom chromatograms), correspond to endogenous HETEs and internal standard 19-HETE-d₃ (5 ng/ml), respectively.

Figure 3. Urinary excretion of 16-, 17-, 18-, 20-HETEs in normal subjects and in cirrhotic patients with and without ascites. *P < 0.05 compared to controls. White bars, controls; striped bars, nonascitic patients; black bars, ascitic patients.

Figure 4. Correlation of RPF (ml/min) and 20-HETE urinary excretion (ng/min) for normal subjects (■), and subjects having cirrhosis with (●) and without (▲) ascites. r = −0.61; P < 0.01.
tained for technical reasons. Nonetheless, the 20-HETE excretion rates for these subjects clearly separated normal from cirrhotic subjects. 20-HETE values for the three subjects with cirrhosis for which RPVs were not available were 4.42, 9.91, and 6.64 ng/min vs. 1.17 and 1.45 ng/min for the two normal subjects. Neither female subjects with cirrhosis (4 of 16) nor female control subjects (3 of 8) differed from corresponding male subjects in terms of HETE excretion. For example, the range of 20-HETE excretory rates for female vs. male patients with cirrhosis was 2.3–18.4 ng/min and 0.3–29.0 ng/min, respectively; and for female vs. male control subjects 1.1–2.2 ng/min and 0.7–2.2 ng/min, respectively.

Urinary excretion of PGE2, PG12, and TxA2 (measured as their hydrolysis products, 6-keto-PGF1α, and TxB2, respectively), was significantly increased only in cirrhotic patients with ascites (Fig. 5), the largest increase over control, (eightfold) occurring for TxB2. In contrast to 20-HETE, however, changes in RPV for cirrhotic subjects were not significantly correlated with excretion of TxB2 (r = −0.40; P > 0.05). Urinary excretion of prostaglandins was not different in male and female subjects. The range of PGE2 excretory rates for female vs. male subjects with cirrhosis was 1.3–3.2 ng/min and 0.2–4.0 ng/min, respectively; for female vs. male control subjects 2.1–2.3 ng/min and 0.6–2.1 ng/min, respectively.

Discussion

The key finding in this study was the selective increase in urinary excretion of 20-HETE in subjects with cirrhosis when compared to 16-, 17-, and 18-HETEs, and a further increase in the excretion of 20-HETE with the development of ascites. Urinary excretion of the subterminal HETEs: 16-, 17-, and 18-HETEs (38). Our study is the first report of the excretion of 16-, 17-, and 18-HETEs by humans, and confirms the important observation of Prakash et al. (31) that 20-HETE is excreted primarily as the glucuronide conjugate, as were the 16-, 17-, 18-, and 19-HETEs. Thus, measurement of CYP-derived HETEs in the urine requires incubation of samples with β glucuronidase, and yielded values in normal subjects comparable to those reported by Prakash et al. (31). The quantity of 20-HETE excreted per unit time in cirrhotic subjects with ascites exceeded that of prostanooids by a factor of three- to five-fold. The excretory rate of TxB2, the stable hydrolysis product of TxA2, that demonstrated the largest increase in the cyclooxygenase products in subjects with cirrhosis and ascites, was less than one-third that of 20-HETE.

Urinary 20-HETE levels probably reflect renal synthesis, Carroll et al. measured efflux of 20-HETE as well as 16-, 17-, 18- and 19-HETEs from the rabbit isolated kidney (32). In the rabbit, AII increased renal efflux of CYP-related HETEs, 20-HETE exhibiting a seven-fold increase. The finding that AII selectively increased renal efflux of HETEs in the rabbit provides a clue as to a possible determinant of enhanced excretion of 20-HETE in cirrhosis, namely, activation of the renin-angiotensin-aldosterone axis, a feature of cirrhosis with ascites (3). PRA, however, was only marginally elevated in cirrhotic subjects with ascites, although conditions that would promote activation of PRA and accentuate differences between cirrhotic subjects and normal subjects were minimized. Thus, PRA was measured on blood samples drawn in the supine position, which is associated with a 50% or greater reduction in PRA (39). An additional consideration, regarding hormonal stimulation of 20-HETE production, is based on the findings of Moore et al. that endothelin plasma levels were greatly elevated in liver disease, particularly in patients with the hepato-renal syndrome (40). We have recently reported that a cytochrome P450 product, probably 20-HETE, participates in the renal vasoconstrictor action of ET-1 (41). ET-1 evoked a three to fourfold increase in efflux of 20-HETE from the rat kidney, and inhibition of CYP-dependent AA metabolism greatly reduced the renal vascular response to ET-1. Thus, 20-HETE may contribute to the renal vasoconstrictor responses to both ET-1 and AII. 20-HETE, in concentrations of 10 nM, contracted renal afferent arterioles (42). The vasoconstrictor properties of 20-HETE (43), its major site of synthesis in the renal vasculature (28), and its release by AII and ET-1 (32, 41), argue for its participation in the renal functional abnormalities associated with cirrhosis, particularly the initial reduction in RPF and the later depression of GFR.

Conjugation of endogenous compounds with glucuronic acid, catalyzed by microsomal glucuronoyl transferase, converts lipid-soluble hormones such as steroids into glucuronides, which can be more easily excreted (44). The glucuronide conjugate is usually biologically inactive (44), which appears to be the case for the 20-HETE glucuronide conjugate, as it was shown to be devoid of renal vasoactivity (M.A. Carroll, Department of Pharmacology, New York Medical College; personal communication). Moreover, the renal proximal tubules as well as the liver demonstrate high specific activity of glucuronoyl transferase (45).

Infusion of 20-HETE has been reported to promote diuresis and natriuresis (22), effects at odds with the pathophysiology of fatty acids including AA, the latter resulting in formation of 20-
of hepatic cirrhosis. The urinary levels of CYP-related HETEs in subjects with cirrhosis, achieving a major vasoconstrictor eicosanoid, is selectively elevated amongst sites other than the nephron were responsible for the large efflux of 20-HETE to the intact kidney, produced a sevenfold increase in renal efflux of 20-HETE (32), suggesting that sites other than the nephron were responsible for the large efflux of 20-HETE in response to AII. More to the point, the capacity of AII to release large quantities of 20-HETE from preglomerular arterioles was reported recently (47). In view of the large capacity of renal arterioles and arteries to produce 20 HETE (42), it is reasonable to consider the renal preglomerular vasculature as a major determinant of reabsorption in deep nephrons (49).

In conclusion, urinary excretion of 20-HETE, a potent renal vasoconstrictor eicosanoid, is selectively elevated amongst the CYP-related HETEs in subjects with cirrhosis, achieving its highest levels in the presence of ascites. The urinary levels of 20-HETE exceeded by threefold those of TxB, the major urinary prostanooid product in cirrhotic subjects. In view of the multiplicity of hormonal and neurohumoral derangements in cirrhosis, it seems likely that, as indicated by Epstein, the renal functional abnormalities require the participation of several hormonal and/or neural effectors, acting in concert (1). The present study has identified 20-HETE as a possible effector of the renal functional abnormalities associated with progression of hepatic cirrhosis.

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