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11,12-EET increases porto-sinusoidal resistance and may play a role in endothelial dysfunction of portal hypertension

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ABSTRACT

CYP450-dependent epoxyeicosatrienoic acids (EETs) are potent arterial vasodilators, while 20-hydroxyeicosatatraenoic acid (20-HETE) is a vasoconstrictor. We evaluated their role in the control of portal circulation in normal and cirrhotic (CCl_4 induced) isolated perfused rat liver. Phenylephrine (PE) and endothelin-1 (ET-1) increased portal perfusion pressure, as did arachidonic acid (AA), 20-HETE, and 11,12-EET. Inhibition of 20-HETE with 12,12-dibromododecenoic acid (DBDD) did not affect basal pressure nor the responses to PE, ET-1, or AA. However, inhibition of epoxygenase with miconazole caused a significant reduction in the response to ET-1 and to AA, without affecting neither basal pressure nor the response to PE. Hepatic vein EETs concentration increased in response to ET-1, and was increased in cirrhotic, compared to control, livers. 20HETE levels were non-measurable. Miconazole decreased portal perfusion pressure in cirrhotic livers. In conclusion, 20HETE and EETs increase portal resistance; EETs, but not 20-HETE, mediate in part the pressure response to ET-1 in the portal circulation and may be involved in pathophysiology of portal hypertension.

1. Introduction

Increased resistance to portal flow plays a major role in the pathogenesis of portal hypertension of cirrhosis [1]. Resistance to portal flow is increased not only because of anatomical alterations, but also because of increased vascular tone [2]. Various vasoconstrictors, like norepinephrine, angiotensin II [3], endothelin-1 (ET-1), are increased in cirrhosis, and upregulation of both ET-A and ET-B receptors has also been shown in hepatic stellate cells [4]. Furthermore, endothelium-dependent vasodilators are probably deficient in the liver, since endothelial NO production is decreased because of post-transcriptional down-regulation of NOS [5]. Among vasoactive systems, cytochrome P450-dependent (CYP450) arachidonic acid (AA) metabolites play an important role in the control of different vascular beds, like the kidney, the heart, the brain, and the mesenteric circulations [6–10]. These compounds have been shown to affect vascular tone directly and indirectly, interacting with other vasoactive systems, like endothelins [11] and nitric oxide [12]. Increased urinary excretion of the potent vasoconstrictor, 20-hydroxyeicosatetraenoic acid (20-HETE), has been demonstrated in patients with cirrhosis [13]. The liver is the richest organ in cytochrome P450 [14], and the highest production is that of the potent arterial vasodilators epoxyeicosatrienoic acids (EETs) [15]. Recent data have shown that these substances are the most powerful known vasodilators in the coronary circulation [6,7]. No information is available on the role of P450-dependent AA metabolites in the control of porto-sinusoidal circulation and in the pathophysiology of portal hypertension. We evaluated the role of P450-dependent AA metabolites, 20-HETE and the EETs, in the control of isolated perfused porto-hepatic circulation from normal and cirrhotic rats.

2. Materials and methods

Male Sprague–Dawley rats were used. All the animal experiments followed an institutionally approved protocol in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996).

2.1. Cirrhosis induction

Cirrhosis was induced in animals (approx. 150 g b.w.) by combined treatment with CCl_4 and phenobarbital, the latter given in drinking water (1.75 g/l) [16]. After 1 week of phenobarbital administration, CCl_4 was given by gavage, starting with a dose of 30 μl,

Abbreviations: EET, epoxyeicosatrienoic acid; 20-HETE, 20-hydroxyeicosatetraenoic acid; AA, arachidonic acid; CYP450, cytochrome P450; PE, phenylephrine; ET, endothelin; DBDD, dibromododecenoic acid; CCl4, carbon-tetrachloride; COX, cyclooxygenase.

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and then increasing doses were administered according to changes in body weight as described by Proctor et al. [17]. CCl_4 was administered once a week and then twice a week for 8–10 weeks. Treatment was terminated 1 week prior to conducting experiments. Rats on phenobarbital during the time of treatment were used as controls. Free access to standard chow was allowed throughout the study. In all the CCl_4-treated rats used for study, portal pressure was measured by a Statham transducer after direct cannulation of the portal vein before the experiments; rats with portal pressure <8 mmHg were excluded from the study.

2.2. Isolated perfused liver preparation

Rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally). After midline and transverse subcostal incisions, the portal vein was cannulated with an 18-gauge cannula and connected to a Statham transducer to determine portal pressure. Once portal pressure was recorded, the cannula was connected to a roller pump. The hepatic artery was tied, as was the inferior vena cava, just above the renal veins. The hepatic veins were cannulated through the right atrium and the inferior vena cava to collect the effluent. The portal vein was perfused with oxygenated (95% O_2, 5% CO_2) Krebs–Henseleit solution, pH 7.4 at 37 °C and a flow rate of 8 ml/100 g b.w./min. In a pilot study, the bile was collected via PE10 tubing and a flow rate >8 μl/min/100 g b.w. was always present. Pressure was measured by a Statham transducer and continuously recorded with a computerized system.

2.3. Responses to agonists and inhibitors

After an initial 30-min basal period, concentration response curves were constructed with PE, -8 to -5 log mol; and ET-1, -12 to -10 log mol, in the presence and absence of 20-HETE synthesis inhibition with 12,12-dibromododec-11-enoic acid (DBDD), 2 μM, or of epoxygenase inhibition with miconazole, 1.0 μM. The doses used were chosen based on our experience and according to Chu et al. [18] and Wang et al. [19]. The maximum response was obtained within 60 s; perfusion pressure returned to baseline values in 2–3 min after PE and 10–15 min after ET-1. Increasing doses were added after return to the baseline pressure. Experiments performed to determine the response to 20-HETE, 11,12-EET were done using three doses: 10–50–100 μmol in 30 s, with and without inhibition of cyclooxygenase (COX) with indomethacin (2.8 μM). Experiments performed to determine the effect of DBDD, miconazole, and indomethacin on the response to arachidonic acid (AA) were also done using the same doses.

2.4. Generation of CYP-dependent AA metabolites by the liver

Levels of cytochrome P450-dependent AA metabolites, 20HETE and EETs (8,9-, 11,12-, and 14,15-EET) were measured in the liver effluent by gas-chromatography–mass-spectrometry, after extraction with ethyl acetate and reverse phase HPLC, as previously reported [13,20]. After addition of deuterated (d2) 20-HETE, 8-9EET, 11-12-EET, 14-15-EET as internal standards, 22–28 ml samples from liver effluents were acidified to pH 3.5, eicosanoids were extracted with ethyl acetate, purified by reverse phase HPLC, using a linear gradient from acetonitrile:water:acetic acid (62.5:37.5:0.05%) to acetonitrile (100%) over 20 min at a flow rate of 1 ml/min, and quantitated by negative chemical GC/MS after derivatization. Pentafluorobenzyl (PFB) esters were prepared by the addition of α-bromo-2,3,4,5,6-penta-fluorotoluene (5 μl) and N,N-diisopropylethylamine (5 μl) to a sample dissolved in acetonitrile (100 μl), and the derivatization was continued at room temperature for 30 min. Trimethylsilyl (TMS) ethers of hydroxyls were prepared by dissolving the sample in N,O-bis(trimethylsilyl)trifluoroacetamide (80 μl) and the reaction was continued at room temperature for 30 min. To separate subterminal P450-HETEs and -EETs, samples were dissolved in iso-octane and 1 μl aliquots were injected into GC column using a temperature program ranging from 150 °C to 300 °C at a rate of 10 °C/min. Methane was used as a reagent gas at a flow resulting in a source pressure of 1.3 torr and the MS was operated in electron capture chemical ionization mode. The endogenous P450-HETEs and -EETs were identified (ion m/z 391) by comparison of GC retention times with authentic P450-HETE standards and quantitated by calculating the ratio of abundance with D2-20-HETE (m/z 393) and d2-EETs.

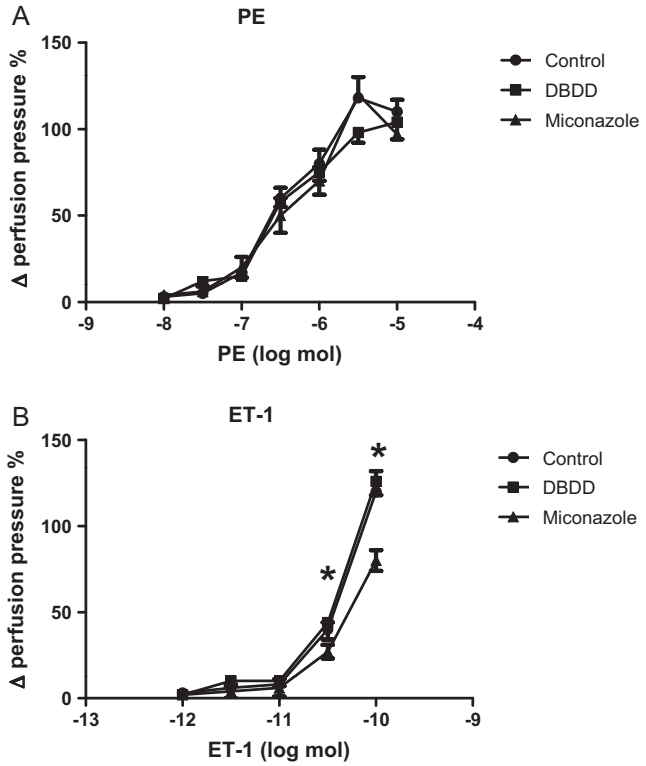

Fig. 1. Pressure response to bolus injections of phenylephrine (PE) (A) and endothelin-1 (ET-1) (B) in isolated perfused livers from normal ($n = 12$) rats, before and after inhibition of 20-HETE synthesis with DBDD (2 μM) and of epoxygenase with miconazole (1 μM). *$p < 0.01$.

2.5. Statistical analysis

Results were expressed as means ± S.E.M. Concentration-response data were analyzed by two-way analysis of variance. Differences between groups were evaluated by unpaired Student's t-test. The effects of the inhibitors on the action of vasoconstrictors were evaluated by paired t-test. Statistical significance was set at $p < 0.05$.

3. Results

In the isolated perfused normal liver the vasoconstrictive effect of PE and ET-1 on portal circulation was not influenced by inhibition of 20-HETE synthesis with DBDD (Fig. 1A and B). Unexpectedly, inhibition of EET synthesis with miconazole significantly reduced vasoconstriction to ET-1, but not to PE (Fig. 1A and B).

As expected, 20-HETE caused vasoconstriction of the portal circulation (Fig. 2), which was COX-dependent, as it was inhibited by indomethacin. Surprisingly, also 11,12-EET caused vasoconstriction in the porto-hepatic circulation (Fig. 2). The effect of

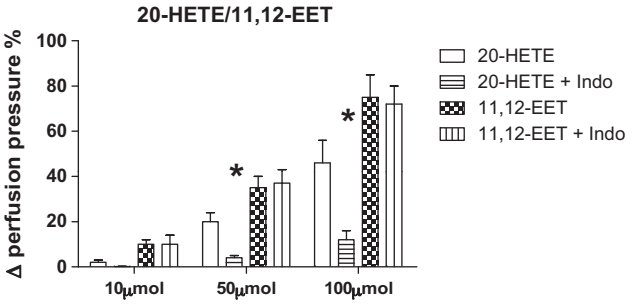

Fig. 2. Effects of different doses of 20-HETE and 11,12-EET, in the presence and absence of COX inhibition with indomethacin (indo), on portal perfusion pressure in isolated perfused livers from normal rats ($n = 5$). *$p < 0.01$ vs. 20-HETE.

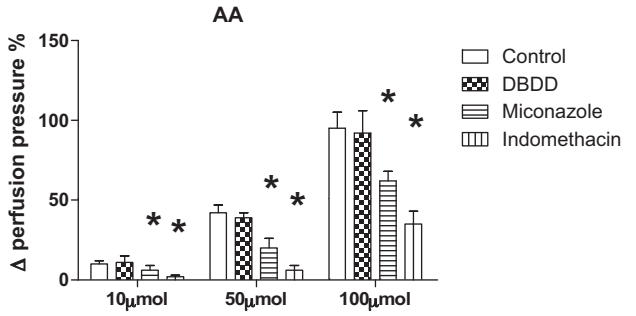

Fig. 3. Effects of different doses of arachidonic acid (AA) on portal perfusion pressure of livers from normal rats ($n = 6$), before and after inhibition of 20-HETE synthesis with DBDD (2 μM), of epoxygenase with miconazole (1 μM), and of COX with indomethacin (2.8 μM). *$p < 0.01$.

11,12-EET was not affected by indomethacin and was similar to that of 14,15-EET (data not shown). AA caused an increase in portal perfusion pressure, which was inhibited by about 60% by indomethacin (Fig. 3). Inhibition of EETs with miconazole decreased the vasoconstricting effect of AA by 40% (Fig. 3), while inhibition of 20-HETE did not have any effect.

20-HETE levels in the liver effluent were below the threshold for measurement by GC/MS, and did not increase after PE and ET-1. EETs levels in the liver effluent were significantly increased by ET-1, but not PE, infusion, and were decreased by miconazole, but not by DBDD (Fig. 4).

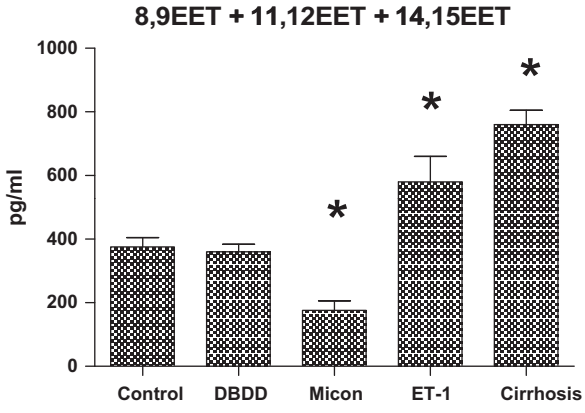

Fig. 4. Concentration of EETs (8,9-EET + 11,12-EET + 14,15-EET) in the liver effluent from normal rats ($n = 8$) before and after miconazole (1 μM) (micon), DBDD (2 μM), ET-1 (100 μmol), and from cirrhotic rats ($n = 8$). *$p < 0.01$ vs. control.

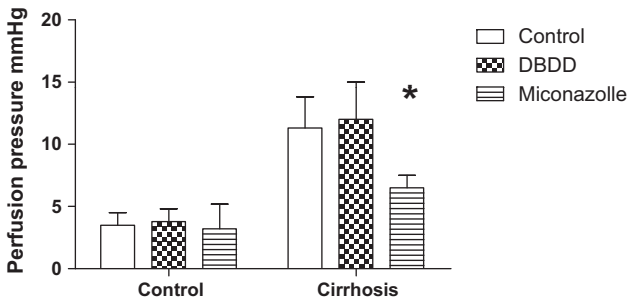

Fig. 5. Effect of inhibition of 20-HETE synthesis with DBDD (2 μM) and of epoxygenase with miconazole (1 μM) on portal perfusion pressure in normal ($n = 12$) and cirrhotic rats ($n = 8$). *$p < 0.01$.

3.1. Cirrhotic rats

Portal pressure (13.3 ± 2.1 vs. 2.5 ± 3 mmHg; $p < 0.001$), as well as portal perfusion pressure (11.3 ± 2.5 vs. 3.5 ± 1.0 mmHg; $p < 0.001$) in the isolated liver were significantly increased in cirrhotic animals. Levels of EETs in the liver effluent were significantly increased in cirrhotic livers and after ET-1, while they were decreased by miconazole (Fig. 4). Inhibition of EETs with miconazole significantly decreased portal perfusion pressure (Fig. 5), while inhibition of 20-HETE was without any effect.

4. Discussion

The present study has evaluated the role of CYP450-dependent AA metabolites in the control of porto-hepatic circulation and in the pathophysiology of portal hypertension of cirrhosis. This is the first demonstration of a vasoconstricting action of 20-HETE and EETs (11,12-EET) in the portal circulation, and of a role of increased EETs in the increase in resistance to portal flow in cirrhosis, opening new perspectives in the treatment of portal hypertension.

Portal hypertension represents the main complication of cirrhosis, as it is the common pathophysiological determinant for the development of esophageal varices, ascites, renal failure, and hepatic encephalopathy [1]. In cirrhosis, portal pressure is the result of increase in both resistance to portal flow and portal inflow [1]. Since the study of Bhathal [21] has demonstrated that porto-hepatic resistance in the cirrhotic liver can be reduced pharmacologically, a lot of studies have evaluated the differential role of increased vasoconstrictors and/or decreased vasodilators in the pathophysiology of portal hypertension [22]. COX metabolites of AA have been shown to increase perfusion pressure in cirrhosis. Graupera et al. [23] have shown that COX inhibition reverses the paradoxical vasoconstricting response to acetylcholine in cirrhotic livers. Gracia-Sancho et al. [24] have shown that the vasoconstricting response to AA in the portal circulation is increased in cirrhotic livers but the COX-1 inhibitor SC-560 and the PGH2/TxA2 receptor antagonist SQ 29,548 could reverse this hyper-response. The liver is the richest organ in cytochrome P450, and can metabolize AA to 20-HETE and the EETs, but no information was available on the role of these vasoactive compounds in the control of liver circulation. Our data demonstrate that 20-HETE is an indomethacin-dependent vasoconstrictor in the porto-hepatic circulation. The EETs are known to cause vasodilatation in various arterial vascular beds and hyperpolarize vascular smooth muscle cells [25,26]. It was a big surprise for us to show that 11,12-EET was a vasoconstrictor in the porto-sinusoidal circulation. A vasoconstricting effect of EETs has been shown only in pulmonary arterial circulation [27], where the effect was cyclooxygenase-dependent, while in the liver it was not affected by indomethacin. Keserü et al. [28] have recently shown that EET-induced pulmonary contraction involves a TRPC6-

dependent pathway, which has not been considered in this study, and it is not known whether and how it affects the tone of portal venules or sinusoids. EETs were increased in the liver effluent of cirrhotic animals and their inhibition with miconazole was able to reduce portal perfusion pressure, while in normal animals it was without any effect. If these results will be confirmed in humans, a new approach to treatment of portal hypertension could be initiated. We evaluated the role of P450-dependent AA metabolites in the response to different vasoconstrictors of the portal circulation: PE and ET-1, and results confirmed the evidence for a vasoconstricting action of EETs. The response to PE was not influenced neither by inhibition of 20-HETE with DBDD, nor of EETs with miconazole, but the response to ET-1 was not affected by DBDD, and was significantly reduced by about 30% by miconazole. The importance of these compounds in the liver was further confirmed by the evidence that AA, when infused into the portal circulation, was causing vasoconstriction, which was significantly reduced by miconazole, but not by DBDD. Both miconazole and DBDD are not specific inhibitors, but with all the limitations due to the possible interaction with other systems, at the concentrations used in this study, according to Chu et al. [18] and Wang et al. [19], they can be considered inhibitors of epoxygenase and w-hydroxylase, respectively, and to this purpose they have been widely used.

We had demonstrated that 20-HETE urinary excretion was increased in patients with cirrhosis, and correlated with renal vasoconstriction in these patients [13]. As 20-HETE has been demonstrated to mediate in part the effect of ET-1 in the kidney [11], and increased amounts of 20-HETE could have come from the liver, we had hypothesized that hepatic production of 20-HETE could be increased in cirrhosis. The results we obtained make us change our hypothesis and consider that increased urinary 20-HETE is not of hepatic origin, unless 20HETE is immediately glucuronated, and thus inactivated, by the liver. On the other side, EETs, acting as endothelium-dependent vasoconstrictors, may play an important role in portal endothelial dysfunction of cirrhotic portal hypertension.

In conclusion, CYP450-dependent AA metabolites, 20-HETE and EETs, are involved in the control of portal circulation, acting as vasoconstrictors. In cirrhosis, increased EETs in the portal circulation appear to participate in the pathophysiology of portal hypertension.

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