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Increased EETs participate in peripheral endothelial dysfunction of cirrhosis

David Sacerdoti^{a,*}, Despina Mania^a, Houli Jiang^b, Paola Pesce^a, Silvia Gaiani^a, Angelo Gatta^a, and Massimo Bolognesi^a

^a Department of Clinical and Experimental Medicine, University of Padova, Via Giustiniani 2, 35100 Padova, Italy

^b Department of Pharmacology, New York Medical College, Valhalla, NY, USA

Abstract

The hyperdynamic circulation of cirrhosis participates in the pathophysiology of portal hypertension. P450-dependent epoxyeicosatrienoic acids (EET) are potent vasodilators. We evaluated plasma levels of EETs in cirrhotic patients and the effect of epoxygenase and nitric oxide synthase (NOS) inhibition on skin blood flow, measured by laser Doppler flowmetry, in normal subjects and cirrhotic patients with and without ascites. Free plasma EETs were increased in cirrhotic patients compared to normal subjects, while the ratio between 8,9-, 11,12-, and 14-15-EET was the same. In cirrhotic patients without ascites, skin blood flow was significantly increased compared to normal subjects. In patients with ascites skin blood flow was significantly reduced compared to control subjects and patients without ascites. Inhibition of epoxygenase with miconazole and of NOS with L-NG-Nitroarginine methyl ester (L-NAME) decreased basal skin flow in normal subjects and in cirrhotic patients, the effect being higher in cirrhotic patients. Miconazole caused a further decrease in flow when administered with L-NAME, both in normal subjects and in cirrhotic patients. In conclusion, EETs participate in the control of peripheral circulation of normal subjects and in the pathophysiology of peripheral vasodilatation of cirrhotic patients with ascites.

Keywords

Cirrhosis; Hyperdynamic circulation; Portal hypertension; Arachidonic acid; Cytochrome P450; Epoxyeicosatrienoic acids (EET); Laser Doppler flowmetry; Skin blood flow; Nitric oxide

1. Introduction

The hyperdynamic circulation of patients with cirrhosis is characterized by high cardiac output, hypervolemia, reduced systemic vascular resistance [1]. These hemodynamic alterations participate in the pathophysiology of portal hypertension which is responsible for the complications of cirrhosis: ascites, hepatorenal syndrome, esophageal varices, encephalopathy.

* Corresponding author. Tel.: +39 049 8212300; fax: +39 049 8754179. david.sacerdoti@unipd.it (D. Sacerdoti)..

Vasodilatation is present mainly in the mesenteric circulation of cirrhotic patients [2], while peripheral vasodilatation is still debated [3–9]. The alterations of peripheral circulation have been studied with different techniques giving different results. Most studies were performed by plethysmography showing an increase in flow to the limbs [4,5,9,10], and a decrease in resistance, which correlate with the severity of the disease [10]. Maroto et al. [7], using the Doppler technique, have shown that brachial and femoral flows are not changed in patients with compensated cirrhosis, while they decrease in severe patients with ascites and renal failure. Also the response of the peripheral circulation to vasoconstrictors, like angiotensin II or noradrenaline, is reduced in cirrhotic patients, as shown by Ryan et al. [9]. Even more contradictory are data on muscular and skin hemodynamics. Lunzer et al. [8] have shown that muscular flow is not altered in cirrhosis, while Thomson et al. [11] and Caraceni et al. [3], with different techniques, have shown vasodilatation of muscle districts. The mechanism responsible for vasodilatation is probably multifactorial and still not completely understood. Most recent studies attribute vasodilatation to endothelial dysfunction due to an excess synthesis and/or release of vasodilators like nitric oxide (NO), prostacyclin (PGI₂), carbon monoxide (CO), cannabinoids, etc. [2].

Among vasodilators involved in the control of circulation, cytochrome P450-dependent epoxyeicosatrienoic acids (EET) play a pivotal, although not well established, role. They are potent vasodilators of the arterial circulation, particularly of coronary arteries [12]. The role of EETs in the control of peripheral vascular tone is not known. These compounds are endothelium-dependent hyperpolarizers [13], involved also in mesenteric vasodilatation of experimental cirrhosis [14]. Increased EETs in the liver have recently been associated with portal hypertension, due to an unexpected vasoconstricting effect on porto-sinusoidal circulation [15]. Increased EETs may be responsible for endothelial dysfunction and peripheral vasodilatation of cirrhosis. Thus, we aimed at evaluating the role EETs in the alterations of peripheral circulation of cirrhosis of different severity. We have focused on the cutaneous circulation, evaluated by the laser Doppler technique, as it allows the *simultaneous* evaluation of different agonists and antagonists in the same patients, by their local administration.

2. Patients and methods

Twenty-eight patients with cirrhosis (post-hepatitis-C in 24, post-hepatitis-B in 4 patients; 18 males and 10 females) of different severity (without ascites: 14 patients, age: 49 ± 6 yr; with ascites: 14 patients, age 54 ± 10 yr) and 14 normal subjects of similar age (49 ± 9 yr) and gender were studied. Patients with cardiovascular diseases or treated with vasoactive drugs were excluded. An informed consent was obtained from each subject, and the study was approved by the local ethical committee.

In the first group of cirrhotic patients ($n = 16$) and normal subjects ($n = 8$), a blood sample was taken, after an overnight fast, for measurement of EETs levels.

In the second group of cirrhotic patients ($n = 12$) and normal subjects ($n = 6$), after at least a 4 hours fast and 20 min lying on a bed at $21\text{--}24^\circ\text{C}$, a laser probe was applied on the dorsal

face of the 2nd phalanx of the 3rd finger and skin flow, expressed as arbitrary units (PFU), was measured for 5 min. The average value was considered as the basal flow.

After baseline measurement, the nitric oxide synthase (NOS) inhibitor, L-NG-Nitroarginine methyl ester (L-NAME) (100 μ M), was applied by iontophoresis and its effects were evaluated on the same finger, while another probe was measuring flow in another finger in order to exclude changes of flow not due to the inhibitor. The epoxygenase inhibitor, miconazole (10 μ M), was then applied together with L-NAME and the effect of the two inhibitors was evaluated. On the 2nd finger the effect of miconazole alone was then evaluated.

Laser Doppler flowmetry gives a semiquantitative assessment of microvascular blood perfusion, reflecting perfusion of capillaries, arterioles, venules, and dermal vascular plexa [16]. Measurements were done using a PeriFlux laser Doppler flowmetry (LDF) (Periflux system 5000, Perimed, Jarfalla/Stockholm, Sweden).

2.1. Measurement of EETs in plasma

Plasma levels of free EETs were measured by gas chromatography mass spectrometry, after extraction with ethyl acetate and reverse phase HPLC, as previously reported [15]. After addition of deuterated (d8) 8-9EET, 11-12-EET, 14-15-EET as internal standards, 100 μ l of plasma 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 μ l) and N,N-diisopropylethylamine (5 μ l) to a sample dissolved in acetonitrile (100 μ l), and the derivatization was continued at room temperature for 30 min. To separate subterminal P450-EETs, samples were dissolved in iso-octane and 1 μ l aliquots were injected into GC column using a temperature program ranging from 150 $^{\circ}$ C to 300 $^{\circ}$ C at a rate of 10 $^{\circ}$ 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-EETs were identified (ion m/z 391) by comparison of GC retention times with authentic P450-EET standards and quantitated by calculating the ratio of abundance with d8-EETs.

2.2. Statistical analysis

Results were shown as mean $\pm$ SD. Differences among groups were analyzed by Anova and unpaired Student's *t*-test. Statistical significance was set at $p < 0.05$.

3. Results

Plasma free EETs levels (8,9EET + 11,12EET + 14-15EET) were significantly increased in cirrhotic patients compared to normal subjects (Fig. 1), while the ratio between 8,9-, 11,12-, and 14-15-EET was the same (Fig. 2).

In cirrhotic patients without ascites, skin blood flow was significantly increased compared to normal subjects (Fig. 3). In patients with ascites, skin blood flow was significantly reduced compared to control subjects and patients without ascites (Fig. 3).

Inhibition of EET synthesis with miconazole caused a decrease in basal skin flow in normal subjects and in cirrhotic patients. The effect of miconazole was significantly higher in cirrhotic patients compared with control subjects (Fig. 4).

Inhibition of NOS with L-NAME caused a significant decrease in skin flow, similar to that caused by miconazole (Fig. 4). Also the effect of L-NAME was higher in cirrhotic patients than in normal subjects. Miconazole caused a further decrease in flow when applied with L-NAME, both in normal subjects and in cirrhotic patients (Fig. 4).

4. Discussion

The results of this study show for the first time: (1) a role of EETs in the control of peripheral circulation in humans, independent from NO, (2) an increase in EETs plasma levels in cirrhosis, (3) an increase in skin blood flow in cirrhotic patients without ascites and (4) a decrease in those with ascites, and (5) an increased peripheral vasoconstricting effect of EETs inhibition in cirrhotic patients.

EETs are endothelium dependent vasodilators acting as hyperpolarizing compounds [13]. In experimental animals, EETs are potent vasodilators of the coronary circulation [12]. The increase in EETs caused by inhibition of soluble epoxide hydrolase decreases resistance in cerebral vessels [17] and reduces blood pressure in hypertensive rats [18]. Data on the role of EETs in cardiovascular physiology and pathophysiology in humans are very few. Genetic variation in cytochrome P450 epoxigenase genes, CYP2C8-CYP2J2, are associated with a higher risk of nonfatal myocardial infarction and ischemic stroke [19,20]. Our data are the first to show a role of EETs in the control of human peripheral arterial resistance. In normal subjects, inhibition of EET synthesis by miconazole decreased skin flow by about 20%, an effect similar to that of NOS inhibition. In mice it has been shown that EETs-induced vasodilatation is mediated by NO, through activation of eNOS and NO release [21]. From our results it appears that at least part of the effect of epoxigenase inhibition is independent from NO, considering that the effect of L-NAME and miconazole together was 30% higher than that of L-NAME alone. Miconazole is not a specific inhibitor, 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. [22], it can be considered an inhibitor of epoxigenase, and to this purpose it has been widely used.

Endothelial NOS-derived NO is considered the most important vasodilator molecule leading to excessive arterial vasodilatation in the splanchnic and systemic circulation in portal hypertension [23]. Other important vasodilating molecules involved in endothelial-derived arterial vasodilatation include CO, prostacyclin, endocannabinoids and endothelium-derived hyperpolarizing factor [23]. We have previously shown that EETs, together with NO and CO, participate in vasodilatation of mesenteric arterioles in cirrhotic animals, and in the hyper-reactivity to acetylcholine [14]. Furthermore, cirrhotic animals showed also a hyper-

reactivity to EETs, which was related to the increased expression of myoendothelial gap junctions and B-calcium-activated potassium channels [24]. In the human peripheral circulation, NO has been shown to be involved in the hyporeactivity to vasoconstrictors like angiotensin-II and norepinephrine in cirrhosis [9]. Our results show that the vasoconstricting response to NOS inhibition with L-NAME was significantly higher in cirrhotic patients than in normal subjects, confirming the hypothesis that increased NO plays a role in peripheral vasodilatation. Nonetheless, also the vasoconstricting response to miconazole was higher in cirrhotic patients than in normal subjects, suggesting that EETs synthesis is increased in these patients. This hypothesis was confirmed by the finding that free plasma levels of EETs, measured in a group of patients with the same clinical characteristics, was significantly increased compared with normal subjects. This increase in EETs plasma levels and in the vasoconstricting response to miconazole was not different in cirrhotic patients with and without ascites, although skin perfusion was higher in those without ascites. One could have hypothesized that the relative amounts of the different EETs, 8-9EET, 11,12EET, and 14,15EET was changed in ascitic patients with a decrease in skin flow, but the ratio of the three EETs was the same in normal subjects and the two groups of cirrhotic patients. Thus, the decreased flow in ascitic patients cannot be considered related to a decreased production of EETs, or NO, but probably to an increase in vasoconstrictors, like endothelin-1, angiotensin-II or other compounds, or to a decreased cardiac output, as recently suggested [7,25].

In conclusion, EETs participate in the control of peripheral circulation of normal subjects and in the pathophysiology of peripheral vasodilatation of cirrhotic patients without ascites. These results may open new perspectives for the treatment of the hyperdynamic circulation of cirrhosis, improving some of the complications of portal hypertension, like renal failure, ascites formation and the development of esophageal varices.

Abbreviations

EET	epoxyeicosatrienoic acid
AA	arachidonic acid
CYP450	cytochrome P450
ET	endothelin
L-NAME	L-NG-Nitroarginine methyl ester
NO	nitric oxide
NOS	nitric oxide synthase
CO	carbon monoxide

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Fig. 1.

Plasma levels of free total EETs: 8,9EET + 11,12EET + 14,15EET in normal subjects ($n = 8$) and cirrhotic patients with ($n = 8$) and without ascites ($n = 8$). * = $p < 0.05$ vs normal subjects; ** = $p < 0.05$ vs cirrhotic patients without ascites.

Fig. 2.

Ratio of plasma levels of 8,9EET, 11,12EET, 14,14EET in normal subjects and cirrhotic patients without and with ascites.

Fig. 3.

Resting skin blood flow measured by laser Doppler flowmetry in normal subjects ($n = 6$) and in cirrhotic patients without ($n = 6$) and with ascites ($n = 6$). PFU = perfusion units. * = $p < 0.05$ vs normal subjects; ** = $p < 0.05$ vs cirrhotic patients without ascites.

Fig. 4.

Effects of inhibition of epoxygenase with miconazole (10 μ M), of nitric oxide synthase with L-NAME (100 μ M), and of both on skin blood flow measured by laser Doppler flowmetry in normal subjects ($n = 6$) and in cirrhotic patients without ($n = 6$) and with ascites ($n = 6$). Miconazole and L-NAME were administered locally by iontophoresis. * = $p < 0.05$ vs normal subjects; § = $p < 0.05$ vs L-NAME.