HEPATOLOGY

HEPATOLOGY, VOL. 64, NO. 3, 2016

Inhibition of Epoxyeicosatrienoic Acid Production in Rats With Cirrhosis Has Beneficial Effects on Portal Hypertension by Reducing Splanchnic Vasodilation

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In cirrhosis, 11,12-epoxyeicosatrienoic acid (EET) induces mesenteric arterial vasodilation, which contributes to the onset of portal hypertension. We evaluated the hemodynamic effects of *in vivo* inhibition of EET production in experimental cirrhosis. Sixteen control rats and 16 rats with carbon tetrachloride-induced cirrhosis were studied. Eight controls and eight rats with cirrhosis were treated with the specific epoxygenase inhibitor *N*-(methylsulfonyl)-2-(2-propynyloxy)-benzenehexanamide (MS-PPOH; 20 mg/kg/day) for 3 consecutive days. Portal blood flow and renal and splenic resistive indexes were calculated through echographic measurements, while portal and systemic pressures were measured through polyethylene−50 catheters. Small resistance mesenteric arteries were connected to a pressure servo controller in a video-monitored perfusion system, and concentration-response curves to phenylephrine and acetylcholine were evaluated. EET levels were measured in tissue homogenates of rat liver, kidney, and aorta, using an enzyme-linked immunosorbent assay. Urinary Na^+ excretion function was also evaluated. In rats with cirrhosis, treatment with MS-PPOH significantly reduced portal blood flow and portal pressure compared to vehicle (13.6 ± 5.7 versus 25.3 ± 7.1 mL/min/100 g body weight, $P < 0.05$; 9.6 ± 1.1 versus 12.2 ± 2.3 mm Hg, $P < 0.05$; respectively) without effects on systemic pressure. An increased response to acetylcholine of mesenteric arteries from rats with cirrhosis (50% effect concentration -7.083 ± 0.197 versus -6.517 ± 0.73 in control rats, $P < 0.05$) was reversed after inhibition of EET production (-6.388 ± 0.263, $P < 0.05$). In liver, kidney, and aorta from animals with cirrhosis, treatment with MS-PPOH reversed the increase in EET levels. In both controls and rats with cirrhosis, MS-PPOH increased urinary Na^+ excretion. *Conclusion:* In rats with cirrhosis, *in vivo* inhibition of EET production normalizes the response of mesenteric arteries to vasodilators, with beneficial effects on portal hypertension. (HEPATOLOGY 2016;64:923-930)

In liver cirrhosis, the increase in portal pressure is due to the increase of intrahepatic resistance and splanchnic blood flow. Splanchnic vasodilation, which is mainly responsible for splanchnic overflow, is the result of an important increase in local and systemic vasodilators and a vascular hyporesponsiveness to vasoconstrictors.[1,2] Several substances and systems have been proposed as possible mediators in the splanchnic vasodilatation of patients with cirrhosis and portal hypertension, and the roles of nitric oxide,[3,4] carbon monoxide,[5,6] and prostacyclin[7,8] have been well documented. An endothelium-derived hyperpolarizing factor, whose nature is controversial, also has been shown to have a crucial role in this process.[9,10] Epoxyeicosatrienoic acids (EETs) are metabolized from arachidonic acid by cytochrome P-450/epoxygenases. In rats with cirrhosis, 11,12-EET induces nitric oxide/prostaglandin I_2-independent vasodilation of

Abbreviations: ACh, acetylcholine; EET, epoxyeicosatrienoic acid; HR, heart rate; MAP, mean arterial pressure; MS-PPOH, N-(methylsulfonyl)-2-(2-propynyloxy)-benzenehexanamide; PE, phenylephrine; PSS, physiological salt solution; RI, resistance index.

Received November 16, 2015; accepted May 25, 2016.

View this article online at wileyonlinelibrary.com.
DOI 10.1002/hep.28686

Potential conflict of interest: Nothing to report.

mesenteric arteries through an activation of large-conductance calcium-activated potassium channels on the vascular smooth muscle cell membranes and an increase of myoendothelial gap junction expression.[11] Therefore, inhibiting the production of vascular EETs or enhancing their degradation may represent a new therapeutic approach to decrease splanchnic vasodilation and portal hypertension in cirrhosis. The aim of this study was to verify in an experimental model of cirrhosis the *in vitro* and *in vivo* effects of the inhibition of EET production on mesenteric artery resistance, splanchnic hemodynamics, and portal pressure.

Materials and Methods

ANIMALS

The study was performed on 32 adult male Wistar rats (body weight 200-225 g; Charles River Laboratories, Calco, Italy). The experiments were carried out in accordance with the legislation of Italian authorities (D.L. 27/01/1992 116), which complies with European Community guidelines (CEE Directive 86/609) for the care and use of experimental animals. The experimental protocol was approved by the Institutional Animal Care and Use Committee. Cirrhosis was induced with the carbon tetrachloride inhalation method in 16 rats as described. Phenobarbital (0.30 g/L) was added in the drinking water. Treatment was followed for 16 weeks, and animals were free of treatment for 1 week.[6] Then, controls and rats with cirrhosis were further divided into treated and untreated groups (eight rats for each group). The treatment consisted of injection into the tail dorsal vein, for 3 consecutive days, of *N*-(methylsulfonyl)-2-(2-propynyloxy)-benzenehexanamide (MS-PPOH; 20 mg/kg/day; Cayman Chemical, Ann Arbor, MI), a specific epoxygenase inhibitor, dissolved in a 1-mL solution of dimethyl sulfoxide and Krebs. In untreated animals a 1-mL solution of dimethyl sulfoxide and Krebs was also injected for 3 consecutive days.

IN VIVO HEMODYNAMIC STUDIES

One hour after the last dose of MS-PPOH or vehicle, rats were anesthetized with ketamine hydrochloride (100 mg/kg) plus midazolam (5 mg/kg) intraperitoneally. A tracheostomy was performed, and a polyethylene-240 tube was inserted into the trachea to ensure a patent airway. Animals were shaved on the abdomen, and the temperature of the animals was maintained at 37 ± 0.5°C. EchoDoppler evaluation was performed using the Vevo high-resolution *in vivo* microimaging system (VEVO 2100 Visualsonics) equipped with a 13-24 MHz probe. B-mode cine loops of a transverse section of the liver were recorded. Portal vein diameter (millimeters) was measured immediately before the origin of the first portal branch. Doppler evaluation of portal vein time-averaged velocity (millimeters per second) was performed at the same level, and mean portal vein velocity was calculated using the following formula: mean portal vein velocity = portal vein time-averaged velocity × 0.57. Portal blood flow was calculated using the following formula: mean portal vein velocity × (portal vein diameter/2)^2 × 3.14 (cubic millimeters per second). B-mode cine loops of a transverse section of the spleen were recorded, color Doppler was used to identify splenic arteries at the hilum, and Doppler evaluation was performed. Peak velocity and end diastolic velocity were measured, and the resistance index (RI) was calculated using the following formula: RI = (peak velocity - end diastolic velocity)/peak velocity. The mean value of three different measurements was calculated. B-mode cine loops of a transverse section of both kidneys were recorded. Color Doppler was used to identify renal interlobar arteries; then, Doppler analysis of identified arteries flow was performed, placing the sample volume in the renal cortex. Peak velocity and end diastolic velocity were measured, and RI was calculated as described before. The mean value of three different measurements was calculated. All rats were imaged by a single operator. Polyethylene−50 catheters were then

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introduced into the femoral artery to measure mean arterial pressure (MAP; mm Hg) and into the ileocolic vein to measure portal pressure (mm Hg). Heart rate (HR, beats/minute) was also recorded. Blood pressures were registered on a multichannel computer-based recorder (PowerLab; AD Instruments, Colorado Springs, CO). Hemodynamic data were collected after a 20-minute stabilization period.

EVALUATION OF THE RESPONSE TO PHENYLEPHRINE AND ACETYLCHOLINE OF SMALL MESENTERIC ARTERIES

The clamped section of the small intestine was placed in a chilled, oxygenated modified Krebs bicarbonate buffer (physiological salt solution [PSS]) containing 118.5 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.8 mM $CaCl_2$, 25 mM $NaHCO_3$, and 11 mM dextrose. Third-order/fourth-order branches of the superior mesenteric artery (170-350 μm in diameter and 1-2 mm in length) were isolated from surrounding perivascular tissue, removed from the mesenteric vascular bed, and mounted on glass micropipettes in a water-jacketed perfusion chamber (Living Systems Instrumentation, Burlington, VT) in warmed (37°C), oxygenated (95% O_2 and 5% CO_2) PSS. The vessels were mounted on a proximal micropipette connected to a pressure servo controller. Subsequently, the lumen of the vessel was flushed to remove residual blood, and the end of the vessel was mounted on a micropipette connected to a three-way stopcock. After the stopcock was closed, the intraluminal pressure was allowed to increase slowly until it reached 80 mm Hg. The vessel was superfused with PSS (4 mL/minute) at 37°C and gassed with 95% O_2 and 5% CO_2 for a 45-minute period of equilibration.[6] Intraluminal pressure was maintained at 80 mm Hg throughout the experiment. After the equilibration period, the vessels were challenged with phenylephrine (PE), an α1-adrenoreceptor agonist (1 μM). An artery was considered unacceptable for experimentation if it demonstrated leaks or failed to constrict by more than 20% to PE. The presence of a functional endothelium was determined on the basis of a prompt relaxation to acetylcholine (Ach; 1 μM) in the vessel precontracted with PE (1 μM). After a 20-minute superfusion period of the arteries with indomethacin (2.8 μM) and N^G-nitro-L-arginine-methyl-ester (1 mM), the effects of PE and ACh administration were evaluated as variations in the internal diameter of the vessels; the response to increasing doses of PE (10^{-8}-10^{-4} M) was reported as a percent of contraction and that to ACh (10^{-9}-10^{-4} M) as a percent of inhibition of the contraction induced by PE (10^{-6} M). PE and ACh were added to the bath (extraluminal application), and cumulative dose-response curves were generated, with 2-minute to 3-minute intervals between doses. After the PE dose-response test, the tissues were washed with fresh PSS for at least 20 minutes before performing the ACh dose-response test. Vascular diameters were measured with the use of a video system consisting of a microscope with a charge-coupled device television camera (Eclipse TS100-F; Nikon, Tokyo, Japan), a television monitor (Ultrak Inc., Lewisville, TX), and a video measuring system (Living Systems Instrumentation). Only one experiment was performed in each artery.

MEASUREMENT OF EET LEVELS IN LIVER, KIDNEY, AND AORTA

Tissue extracts from liver, kidney, and aorta from untreated controls, rats with cirrhosis, and rats with cirrhosis treated with MS-PPOH (n = 4 for each group) were prepared from frozen tissues; and samples were extracted by radio immunoprecipitation assay lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.25% deoxycholic acid, 1% Nonidet P40, 10 mM ethylene diamine tetraacetic acid). The total protein content of tissue extracts was determined using the Bicinchoninic Protein Assay Kit (Euroclone, Italy).

EET levels were determined in tissue homogenates using an enzyme-linked immunosorbent assay (BlueGene Biotech), according to the manufacturer's instructions. The EET enzyme-linked immunosorbent assay kit applies the competitive enzyme immunoassay technique using a monoclonal anti-EET antibody and an EET-horseradish peroxidase conjugate. Briefly, the assay samples and buffer were incubated together with EET-horseradish peroxidase conjugate in a precoated plate. The plate was then washed and incubated with a substrate for horseradish peroxidase enzyme. Subsequently, the intensity of color was measured spectrophotometrically at 450 nm in a microplate reader. The amount of EETs, according to the reference standard curve, was normalized to the protein content.

SODIUM EXCRETION AND RENAL FUNCTION EVALUATION

Urine was obtained from four controls and four rats with cirrhosis maintained in metabolic cages through a 24-hour collection before the first dose and after the

second administration of MS-PPOH. Urinary Na^+ values were determined using the appropriate analyzers (Aldrich/Sigma, St. Louis, MO). Intracardiac blood samples (0.5 mL) were collected under anesthesia before the sacrifice of the animals. Creatinine values were determined using the appropriate analyzers (Aldrich/Sigma).

DATA ANALYSIS

Data were expressed as mean ± standard deviation. Concentration-response data derived from each vessel were fitted separately to a logistic function by nonlinear regression, and the molar concentrations of PE and ACh causing 50% of the maximal vasoconstrictor and vasorelaxant effect, respectively, were calculated and expressed as log [M]. From the same regression, the maximal contraction and relaxation of the artery were also calculated as percentages of contraction and relaxation (that is, reduction in vessel diameter relative to baseline diameter and increase in vessel diameter relative to the diameter after precontraction with PE, respectively). Data were analyzed by analysis of variance or Student *t* test for paired or unpaired observations when appropriate. The null hypothesis was rejected at $P < 0.05$.

Results

At histology, all rats treated with carbon tetrachloride included in the study had macronodular or micronodular cirrhosis, while control rats had no appreciable alteration in the liver. At the time of the study, no difference in body weight was observed between controls and rats with cirrhosis.

EFFECT OF *IN VIVO* INHIBITION OF 11,12-EET PRODUCTION ON SPLANCHNIC HEMODYNAMICS

Portal blood flow was 21.8 ± 3.9 mL/min/100 g body weight in untreated control rats, 15.7 ± 5.9 mL/min/100 g body weight in control rats treated with MS-PPOH, 25.3 ± 7.1 mL/min/100 g body weight in untreated rats with cirrhosis, and 13.6 ± 5.7 mL/min/100 g body weight in rats with cirrhosis treated with MS-PPOH, significantly reduced compared to untreated rats with cirrhosis ($P = 0.026$). In rats with cirrhosis, splenic RI was lower than in controls (0.40 ± 0.03 versus 0.51 ± 0.08, $P = 0.045$); inhibition of EET production reversed RI in rats with cirrhosis (0.49 ± 0.06, $P = 0.033$ versus untreated rats with cirrhosis, *P* not significant versus control rats). No effect on splenic RI was observed in control rats after MS-PPOH administration (0.51 ± 0.05). No differences in renal RI were observed among groups: 0.46 ± 0.06 in untreated control rats, 0.45 ± 0.07 in treated control rats, 0.47 ± 0.07 in untreated rats with cirrhosis, 0.53 ± 0.08 in treated rats with cirrhosis.

In untreated rats with cirrhosis, as expected, portal pressure was higher compared to controls (12.2 ± 2.3 mm Hg versus 7.3 ± 1.9 mm Hg, $P = 0.003$). Treatment with MS-PPOH significantly reduced portal pressure in rats with cirrhosis (9.6 ± 1.1 mm Hg, $P = 0.016$) (Fig. 1), although a difference from control rats was still present ($P = 0.031$). In control rats, inhibition of EET production did not affect portal pressure (7.1 ± 2 mm Hg). No significant changes in MAP or HR were observed among groups: controls (MAP = 85.7 ± 8.1 mm Hg, HR = 305 ± 37 beats/minute); controls+MS-PPOH (MAP = 86.8 ± 9.8 mm Hg, HR = 305 ± 42 beats/minute); cirrhosis (MAP = 85.1 ± 16.6 mm Hg, HR = 311 ± 41 beats/minute); cirrhosis+MS-PPOH (MAP = 85.4 ± 14.3 mm Hg, HR = 309 ± 36 beats/minute).

RESPONSE TO PE OF SMALL MESENTERIC ARTERIES

The response to PE was comparable in the four groups of rats examined: untreated control rats (50% effect concentration −5.933 ± 0.209), control rats treated with MS-PPOH (−5.927 ± 0.165), untreated rats with cirrhosis (−5.686 ± 0.091), and rats with cirrhosis treated with MS-PPOH (−5.543 ± 0.159). Also, with regard to the maximal concentration, no significant differences were observed (Fig. 2).

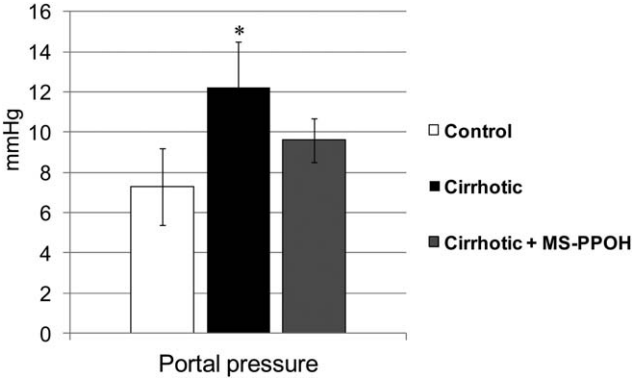

FIG. 1. Portal pressure (mean ± standard deviation) in control, untreated rats with cirrhosis and rats with cirrhosis treated with MS-PPOH. *Significantly different ($P < 0.05$) from the other groups.

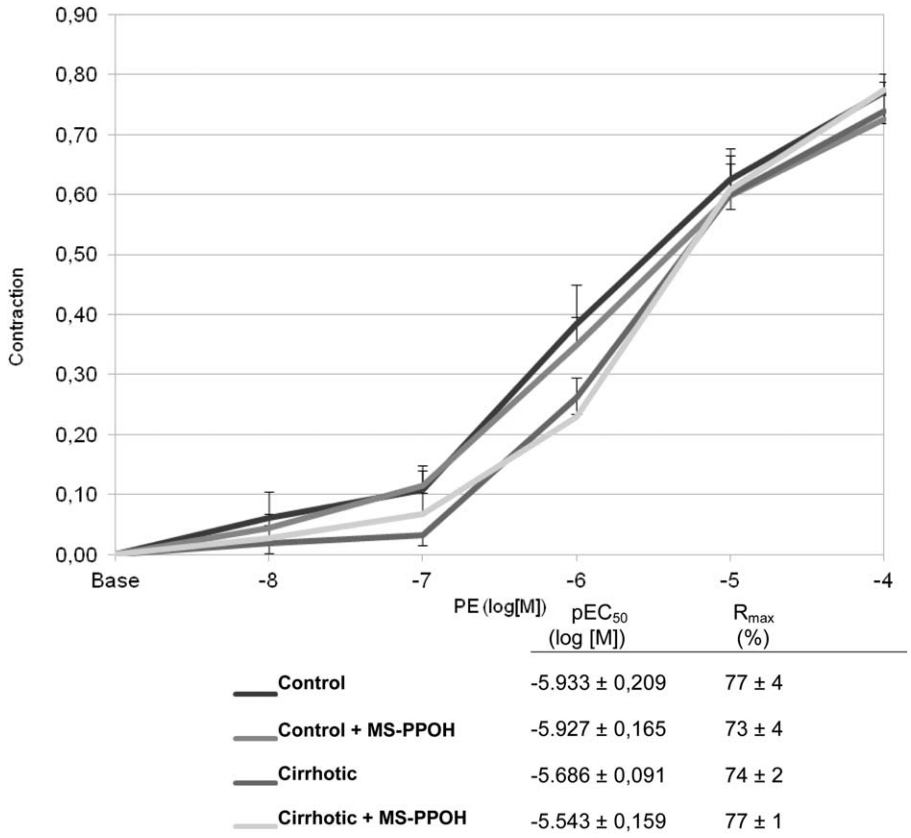

FIG. 2. Dose-response curves for PE of small resistance mesenteric arteries in controls and rats with cirrhosis treated or not with MS-PPOH. Values represent mean ± standard deviation. Abbreviations: pEC_{50}, molar concentrations of PE and ACh causing 50% of the maximal vasoconstrictor and vasorelaxant effects, respectively; R_{max}, maximal relaxation.

RESPONSE TO ACh OF SMALL MESENTERIC ARTERIES

The ACh response was higher in the group of untreated rats with cirrhosis compared to untreated controls (50% effect concentration -7.083 ± 0.197 versus −6.517 ± 0.73, $P = 0.014$), controls treated with MS-PPOH (−6.566 ± 0.109, $P = 0.018$), and rats with cirrhosis treated with MS-PPOH (−6.388 ± 0.263, $P = 0.044$). Comparing maximal relaxation, there was a statistically significant difference between untreated rats with cirrhosis (106 ± 2%) and the other groups: untreated control rats (93 ± 3%, $P < 0.01$), treated control rats (95 ± 2%, $P < 0.01$), treated rats with cirrhosis (95 ± 6%; $P < 0.05$) (Fig. 3).

EET LEVELS IN LIVER, KIDNEY, AND AORTA

In liver, kidney, and aorta from untreated rats with cirrhosis, EET levels were significantly higher than in controls ($P < 0.05$). Treatment with MS-PPOH decreased EET levels in rats with cirrhosis liver, kidney, and aorta ($P < 0.05$) (Fig. 4).

SODIUM EXCRETION AND RENAL FUNCTION

In rats with cirrhosis, natriuresis was slightly lower, but not significantly, than in control rats. In both controls and rats with cirrhosis, urinary Na^+ excretion increased after MS-PPOH treatment ($P < 0.05$; Fig. 5). Serum creatinine concentration was similar in the four groups of rats.

Discussion

In cirrhosis, the increase in splanchnic blood flow, which is determined by an arterial vasodilation in the splanchnic vascular bed, has a major role in the onset of portal hypertension.[12] The mechanisms responsible for the reduction in mesenteric arterial resistance in cirrhosis have been extensively investigated. Nitric oxide is likely the main vasodilator involved in this

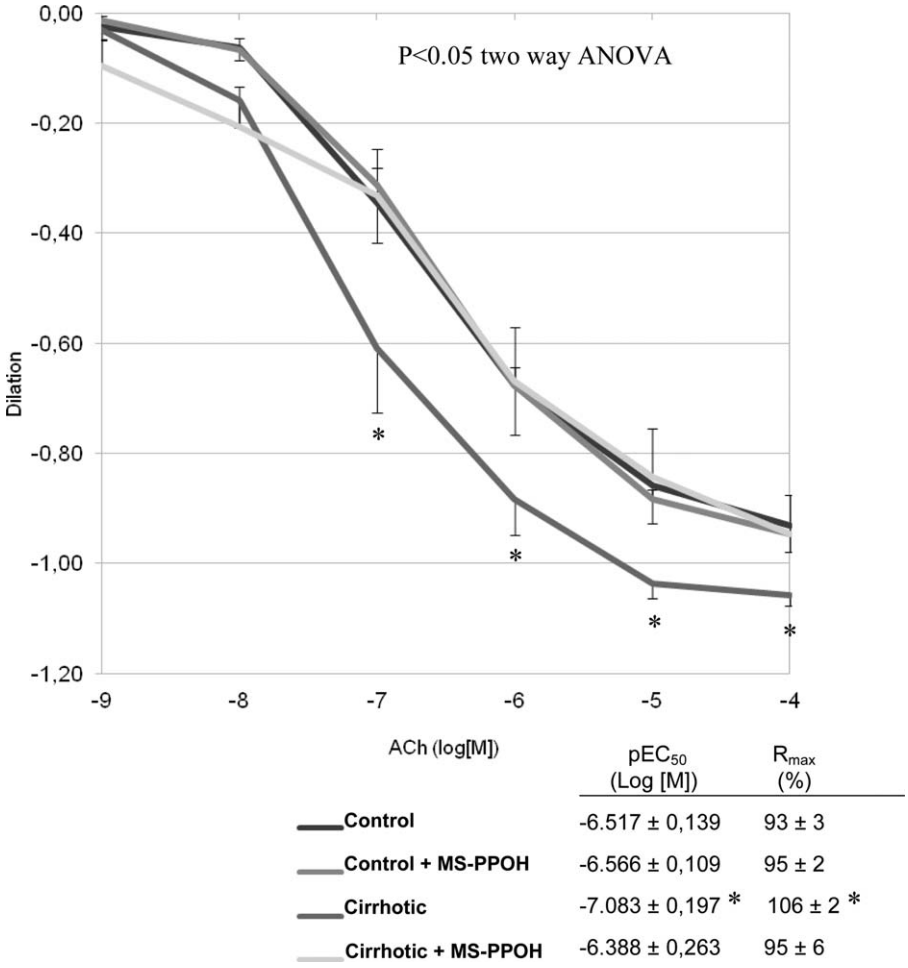

FIG. 3. Dose-response curves for ACh of small resistance mesenteric arteries in controls and rats with cirrhosis treated or not with MS-PPOH. *Significantly different ($P < 0.05$) from the other curves. Values represent mean ± standard deviation. Abbreviations: ANOVA, analysis of variance; pEC_{50}, molar concentrations of PE and ACh causing 50% of the maximal vasoconstrictor and vasorelaxant effects, respectively; R_{max}, maximal relaxation.

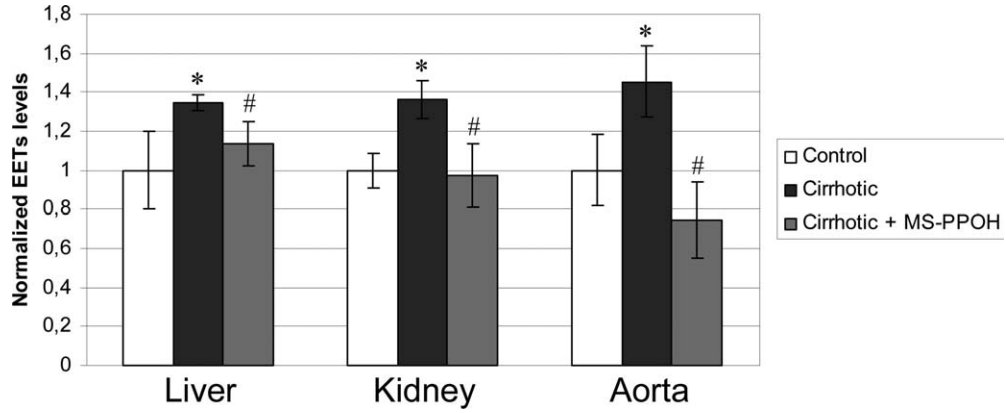

FIG. 4. EET levels in liver, kidney, and aorta from control, untreated rats with cirrhosis and rats with cirrhosis treated with MS-PPOH. Values represent mean ± standard deviation. *$P < 0.05$ versus controls, #$P < 0.05$ versus cirrhosis.

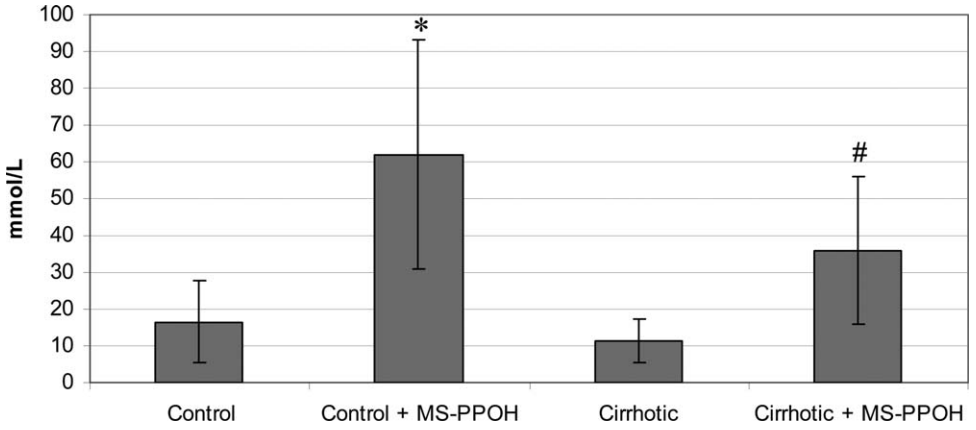

FIG. 5. Na^+ urinary excretion (mean ± standard deviation; millimoles per liter). $^*P < 0.03$ versus controls, $^{\#}P < 0.04$ versus cirrhosis.

process but is not the only factor responsible because nitric oxide synthase inhibition with N^G-nitro-L-arginine-methyl-ester, especially in the advanced stages of the disease, only partially reversed the alterations in splanchnic hemodynamics.[6,13] We have demonstrated that in rats with cirrhosis 11,12-EET plays a major role in the mesenteric arterial vasodilation and that this effect is mediated by an increased myoendothelial gap junction expression.[11] Moreover, we have shown that in patients with cirrhosis EET plasma levels are increased and that the vasoconstricting response to miconazole (another inhibitor, although not specific, of EET production) is higher than in normal subjects.[14] Therefore, we speculated that a modulation of 11,12-EET production may be a novel strategy to reverse portal hypertension and the hyperdynamic syndrome in cirrhosis. To verify this hypothesis, MS-PPOH, a specific epoxygenase inhibitor, was used. Unlike previous studies carried out *in vitro*, in this study we evaluated the effect of MS-PPOH administration also *in vivo*.

This study shows that in rats with carbon tetrachloride-induced cirrhosis the inhibition of EET production reduces portal blood flow and pressure without affecting systemic pressure. This effect is probably ascribable to a reduction in the splanchnic vasodilation of rats with cirrhosis because MS-PPOH administration reversed the increased mesenteric response to ACh. This hypothesis is supported by the fact that in rats with cirrhosis treatment with MS-PPOH normalized the reduced splenic artery RI. MS-PPOH inhibits the production of all EETs, but because in cirrhosis 11,12-EET has been demonstrated to play a key role in the increased mesenteric vasodilation, we can speculate that a decrease in the 11,12-EET bioavailability in the splanchnic district is the main factor responsible for these hemodynamic effects. On the other hand, no significant difference in the response to PE was observed, suggesting that the reduction in 11,12-EET production in the splanchnic vascular system affected only the response to vasodilators, not that to vasoconstrictors. We cannot exclude that in rats with cirrhosis the effect on portal pressure was secondary also to a modulation of intrahepatic resistance because we have observed an inhibitory effect on the production of EETs by MS-PPOH not only in the vascular district but also in the liver. No effects on splanchnic hemodynamics or on the response of mesenteric arteries to PE and ACh were observed in control rats. Therefore, EETs seem to play a role in mesenteric endothelium-dependent relaxation in rats with cirrhosis but not in control rats. This finding is in line with our previous study which demonstrated that the inhibition of epoxygenase with miconazole modified the response to ACh only in rats with cirrhosis,[15] confirming that an imbalance in the axis of EETs in the splanchnic district is a typical feature of cirrhosis.

In this study we also evaluated the effect of MS-PPOH on natriuresis because, in cirrhosis, renal sodium retention can develop.[16-18] Despite the fact that the reduction of renal sodium excretion in animals with cirrhosis was not statistically significant, MS-PPOH administration *in vivo* increased sodium excretion both in controls and in rats with cirrhosis. The natriuretic effect of MS-PPOH is well in keeping with the observations of Brand-Schieber et al.[19] EETs produced in the proximal tubule regulate the activity of

adenosine triphosphatase-Na^+/K^+ and serve as second messengers for the natriuretic effect of dopamine, parathyroid hormone, and angiotensin II.[20] We cannot exclude that in rats with cirrhosis also an increased renal flow following splanchnic vasoconstriction may promote natriuresis, but our data do not support this theory as renal artery RIs were not different among treated and untreated animals, while a reduction in renal EET levels was observed after administration of MS-PPOH. In rats with cirrhosis a natriuretic effect of MS-PPOH has never been shown before.

In conclusion, our data show that in rats with cirrhosis *in vivo* inhibition of EET production has beneficial effects on portal hypertension by reducing splanchnic vasodilation. Modulating EET production may be a novel strategy in the treatment of patients with cirrhosis and portal hypertension.

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