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BASIC STUDIES

Increased myoendothelial gap junctions mediate the enhanced response to epoxyeicosatrienoic acid and acetylcholine in mesenteric arterial vessels of cirrhotic rats

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Abstract

Background: Cirrhotic portal hypertension is characterized by mesenteric arterial vasodilation and hyporeactivity to vasoconstrictors. *Aim:* We evaluated the role of epoxyeicosatrienoic acid (EET) and of myoendothelial gap junctions (GJ) in the haemodynamic alterations of experimental cirrhosis. *Methods:* Thirty-five control rats and 35 rats with carbon tetrachloride (CCl_4)-induced cirrhosis were studied. Small resistance mesenteric arteries (diameter $< 350\,\mu m$) were connected to a pressure servo controller in a video-monitored perfusion system. Concentration–response curves to acetylcholine (ACh) were evaluated in mesenteric arteries pre-incubated with indomethacin, N^G-nitro-L-arginine-methyl-ester and 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one before and after the epoxygenase inhibitor miconazole or 18α-glycyrrhetinic acid (18α-GA) (GJ inhibitor). EC_{50} was calculated. Concentration–response curves to 11,12-EET were also evaluated. mRNA and protein expression of connexins (Cxs) in the mesenteric arteries was evaluated by real-time PCR and immunohistochemistry. *Results:* The ACh response was increased in cirrhotic rats (EC_{50}: -6.55 ± 0.10 vs. -6.01 ± 0.10 log[M]; $P < 0.01$) and was blunted by miconazole only in cirrhotic animals. 18α-GA blunted the response to ACh more in cirrhotic than that in control rats ($P < 0.05$). Concentration–response curves to 11,12-EET showed an increased endothelium-dependent vasodilating response in cirrhotic rats ($P < 0.05$); the BK_{Ca} inhibitor Iberiotoxin (25 nM) blocked the response in normal rats but not in cirrhotic rats, while 18α-GA blunted the response in cirrhotic rats but not in control rats. An increased mRNA and protein expression of Cx40 and Cx43 in cirrhotic arteries was detected ($P < 0.05$). *Conclusions:* The increased nitric oxide/PGI_2-independent vasodilation of mesenteric arterial circulation in cirrhosis is because of, at least in part, hyperreactivity to 11,12-EET through an increased expression of myoendothelial GJs.

In cirrhosis, splanchnic vasodilation is a key mechanism leading to increased portal inflow and contributing to portal hypertension. It is pivotal in the onset of clinical complications such as ascites, spontaneous bacterial peritonitis, hepatorenal syndrome or gastro-intestinal haemorrhage.

A large body of evidence supports the role of nitric oxide (NO) as one of the hallmarks involved in the pathogenesis of vasodilation and vascular hypocontractility in cirrhosis (1). Tumour necrosis factor-α, increased bacterial translocation and shear stress were identified as factors triggering endothelial nitric oxide synthase upregulation (1). On the other hand, other substances/systems also participate in the mesenteric artery hyporeactivity to constrictors in cirrhosis. Indeed, it has been shown that NOS inhibition, alone or combined with cyclo-oxygenase (COX) inhibition, does not entirely suppress splanchnic vasodilation, arterial smooth muscle cell relaxation and hyporeactivity to vasoconstrictors of mesenteric arteries, both in portal hypertensive rats (2, 3) and in cirrhotic rats (4, 5), particularly in the advanced stage of the disease. Therefore, besides NOS/NO and COX/PGI_2, other vasoactive systems are involved (5–9). A role for the endothelium-derived hyperpolarizing

factors (EDHFs) has been hypothesized. EDHFs are factors responsible for the endothelium-dependent relaxation that is insensitive to the inhibition of NOS and COX, mediated by vascular smooth muscle cells (VSMC) hyperpolarization (6, 10) and sensitive to potassium channel blockers (6, 10). Barriere *et al.* (3) demonstrated that in the superior mesenteric artery of cirrhotic rats, an EDHF exists that may replace NOS/COX products to induce endothelium-dependent arterial relaxation. Which molecule constitutes EDHF is controversial (6). The major candidate molecules currently considered are (i) arachidonic acid metabolites [epoxyeicosatrienoic acid (EET)]; (ii) the monovalent cation, K^+; (iii) components of gap junctions (GJs); and (iv) hydrogen peroxide (6).

Endothelial cytochrome P450 generates EETs, which act mainly by opening large-conductance calcium-activated potassium channels (BK_{Ca}) on the VSMC membrane (10–14), and the α subunit of BK_{Ca} is overexpressed in cirrhotic rats (9). EDHF-mediated relaxation could also be promoted by the opening of endothelial potassium channels (small and intermediate conductance Ca^{2+}-activated K^+ channels, SK_{Ca} and IK_{Ca} respectively) (15). The consequent efflux of K^+ from endothelial cells hyperpolarizes VSMC via the activation of inward rectifier potassium channels and Na^+/K^+ ATPase on the VSMC membrane (16, 17). On the other hand, electrical conductance can occur directly from endothelium to VSMC through the GJs (15, 16, 18, 19). Therefore, the endothelial cell hyperpolarization because of the opening of endothelial SK_{Ca} and IK_{Ca} (10) could be transferred directly via the myoendothelial GJ to VSMC with consequent relaxation (16).

The role of GJ may mainly be relevant in small resistance mesenteric arteries, where EDHF-mediated vasodilation is prominent (20) with respect to that mediated by NO (21). Indeed, myoendothelial GJ plaques are more numerous in distal vessels, suggesting a stronger electrical coupling between the endothelium and the media as vessel size diminishes (15).

Some observations suggest that endothelial SK_{Ca} and IK_{Ca} may also be opened by an autocrine effect of EETs (15, 22, 23). Therefore, in some arteries, EETs may act via the endothelium by initiating a hyperpolarizing response (22) that is conducted to the media by myoendothelial GJ (15, 23). The role of EETs and of myoendothelial GJ in splanchnic vasodilation of experimental cirrhosis has not been investigated.

The aim of the study was to investigate the role of EET and GJ in endothelium-dependent vasodilation of small resistance mesenteric arteries in experimental cirrhosis.

Materials and methods

Animals

The study was performed on 70 adult male Wistar rats (Charles River Laboratories, Calco, Italy), body weight 200–225 g. The experiments were carried out in accordance with the legislation of Italian authorities (D.L. 27/01/1992 no. 116), which complies with European Community guidelines (CEE Directive 86/609) for the care and use of experimental animals. All animals received human care. The experimental protocol was approved by the Institutional Animal Care and Use Committee.

Cirrhosis was induced with the carbon tetrachloride (CCl_4) inhalation method in 35 rats drinking phenobarbital (0.30 g/l in drinking water) as described previously (5). Treatment was followed for 16 weeks, and animals were free of treatment for the last week before the experiment. Under anaesthesia with ketamine hydrochloride (100 mg/kg body wt intramuscularly), a mid-ventral laparotomy was performed and a section of the small intestine was removed. Thirty-five age-matched animals were used as controls.

Isolated microvessel preparation

As the low splanchnic vascular resistance observed in portal hypertension depends mostly on mesenteric resistance arteries, which are precapillary arteries with diameters narrower than 500 μm (24), we studied the endothelial function, the response to EET, mRNA and protein expression in small mesenteric resistance arteries. The no-flow model was chosen to avoid interference from shear stress.

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-/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, USA) 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 mmHg. The vessel was superfused with PSS (4 ml/min) at 37 °C gassed with 95% O_2 and 5% CO_2 for a 45-min period of equilibration (5). Intraluminal pressure was maintained at 80 mmHg 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). To remove the endothelium, 2 ml of air was flushed through the lumen

(9, 25). In these arteries, the absence of a functional endothelium was confirmed after preconstriction with PE by the absence of response to ACh with a normal response to sodium nitroprusside, an endothelium-independent vasodilator. The effects of ACh and EET administration were evaluated as variations in the internal diameter of the vessels preconstricted with PE 10 μM; all responses were reported as percent inhibition of the contraction induced by PE. Only one experiment was performed in each artery.

Evaluation of the response to acetylcholine and to 11,12-epoxyeicosatrienoic acid of small mesenteric arteries preconstricted with phenylephrine in carbon tetrachloride cirrhotic rats

Responses to increasing concentrations of ACh (10^{-9}–10^{-4} M) and of 11,12-EET (10^{-9}–10^{-6} M) were determined in arteries superfused with PSS-containing vehicles for the inhibitors tested. Inhibitors were added to freshly prepared PSS, and a 20- to 30-min drug–tissue contact time was allowed before retesting the response to ACh or 11,12-EET in the same vessel. ACh or 11,12-EET was added to the bath (extraluminal application), and cumulative concentration–response curves were generated, with 2- to 3-min intervals between doses. After each concentration–response test, the tissues were washed with fresh PSS for at least 20 min.

Vascular diameters were measured 1–3 min after the addition of ACh or 11,12-EET using a video system consisting of a microscope with a CCD television camera (Eclipse TS100-F, Nikon, Tokyo, Japan), a television monitor (Ultrak Inc., Lewisville, TX, USA) and a video measuring system (Living Systems Instrumentation).

Miconazole, an inhibitor of epoxygenase, which binds to the haeme moiety of cytochrome P450 (26), was used to inhibit EET production. The specificity of imidazole antimycotics for epoxygenases was sometimes questioned. Indeed, a high dosage of these drugs non-selectively inhibits all CYP-450 mono-oxygenases (27). Moreover, a few authors have suggested that cytochrome P450 inhibitors *per se* depolarize the smooth muscle membrane, suppressing the EDHF-mediated responses because of non-specific actions on K^+ channels (26, 28). This problem may also be related, at least in part, to the dosage used. Therefore, we are confident that miconazole, at the dose of 2 μM, in the presence of inhibition of COX and NOS, can be considered a specific inhibitor for epoxygenases.

To inhibit GJs, 18α-glycyrrhetinic acid (18α-GA), a putative GJ uncoupler (21), was used. Indeed, among the derivatives of glycyrrhizic acid, 18α-GA is the most suitable for assessing the contribution of GJ to endothelium-dependent relaxation, because it does not exhibit any direct smooth muscle relaxant activity or any interference with NO activity (15).

To block the effect of NO, we used the NOS inhibitor N^G-nitro-L-arginine-methyl-ester (L-NAME) to inhibit NO production and the soluble guanylyl cyclase (sGC) inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) to inhibit the main effector of NO in VSMC.

In control rats and in cirrhotic rats, we evaluated
(a) concentration–response curves to ACh after a 30-min superfusion of the arteries with the COX inhibitor indomethacin (indo) (2.8 μM), the NOS inhibitor L-NAME (1 mM) and the sGC inhibitor ODQ (10 μM).
(b) the effect of miconazole (2 μM) on concentration–response curves to ACh in arteries treated with COX inhibitor indo (2.8 μM), the NOS inhibitor L-NAME (1 mM) and the sGC inhibitor ODQ (10 μM).
(c) the effect of 18α-GA (100 μM) on concentration–response curves to ACh in arteries treated with the COX inhibitor indo (2.8 μM), the NOS inhibitor L-NAME (1 mM) and the sGC inhibitor ODQ (10 μM).
(d) concentration–response curves to 11,12-EET before and after a 30-min superfusion with the BK_{Ca} inhibitor iberiotoxin (IbTx) (25 nM).
(e) concentration–response curves to 11,12-EET before and after a 30-min superfusion with the GJ inhibitor 18α-GA (100 μM).
(f) concentration–response curves to 11,12-EET in arteries after removal of the endothelium.

Chemicals

11,12-Epoxyeicosatrienoic acid was obtained by Cayman Chemical Company (Ann Arbor, MI, USA). All other chemicals were obtained from Sigma Chemical (St Louis, MO, USA). Indo and ODQ were dissolved in ethanol and then diluted in PSS. 18α-GA was dissolved in DMSO and then diluted in PSS. The other chemicals were dissolved in deionized water and diluted with PSS.

mRNA expression of connexins in mesenteric arteries

After the removal of veins and adipose tissue, small mesenteric arteries (30–40 arteries with diameter < 500 μm) were collected from each rat, snap frozen in liquid N_2 and stored at $-80\,^{\circ}$C until analysed. mRNA expression of Cx37, Cx40, Cx43 and Cx45, which are the predominant isoforms of GJ proteins expressed in the vascular wall (15, 19–31), was evaluated. Total RNA from mesenteric arteries was extracted using RNasy Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and quantified by spectrophotometry at 260 nm. Total RNA (up to 1 μg) was reverse transcribed using Superscript II reverse transcriptase (Invitrogen). The expression of Cxs was determined using SYBR green master mix (Roche Diagnostics GmbH, Indianapolis, IN, USA) and was performed in a LightCycler 2.0 Real-Time PCR System [(Roche Diagnostics GmbH) using the following primers: Cx37 sense: 5′-GGGCACTAGCAGCCATAG AA-3′; Cx37 reverse: 5′-TCGAGAGACGTAGCAGTCCA-3′; Cx40 sense: 5′-GCCTGGCTGAACTCTACCAC-3′; Cx40 reverse: 5′-TGGGCTGTTCTTTAGGCACT-3′; Cx43 sense: 5′-TCCTTGGTGTCTCTCGCTTT-3′; Cx43 reverse:

5′-GAGCAGCCATTGAAGTAGGC-3′; Cx45 sense: 5′-TGT TTGAGGTGGGCTTTCTC-3′; Cx45 reverse: 5′-GAGT CTCGAATGGTCCCAAA-3′]. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase [(*Rattus norvegicus*) sense: 5′-AGACAGCCGCATCTTCTTGT-3′, reverse: 5′-CTTGCCGTGGGTAGAGTCAT-3′] was determined in parallel in all the amplification sets to assess the integrity of the total RNA extracts. The single-tube Real-Time Reverse Transcription-Polymerase Chain Reaction assays were performed using the LightCycler 2.0 Real-Time PCR System (Roche Diagnostics GmbH) and consisted of one denaturation cycle at 95 °C for 10 min, 45 cycles of amplification at 94 °C for 1 s, 60 °C for 10 s and 72 °C for 10 s. Melt curve analysis was performed by ramping products from 45 to 98 °C, acquiring fluorescence readings for each degree change. For Cx genes, the fluorescence of the SYBR green dye was determined as a function of the PCR cycle number, yielding the threshold cycle (C_T) number. The C_T values were used to quantify the PCR product; ΔC_T was calculated by subtracting C_T (control gene: housekeeping) from C_T (target gene: connexin). The ΔC_T mean value of the control rats was used arbitrarily as a constant that was subtracted from all other ΔC_T values to determine the $\Delta\Delta C_T$ value. Samples were run in triplicate and fold changes were generated for each sample by calculating $2^{-\Delta\Delta C_T}$ (32).

Immunohistochemistry

Protein expression of Cx37, Cx40, Cx43 and Cx45 was evaluated in the vascular wall. After the removal of veins and adipose tissue, small mesenteric arteries were collected from each rat. Blood vessels were then fixed with 4% paraformaldehyde in 0.1 mol/l PBS at 4 °C for 1–2 h and then frozen in OCT using a gelatin capsule. Cryosections of 3–4 μm were pre-incubated for 30 min in a blocking solution containing 2% BSA (or 2% of the serum of the secondary antibody), followed by incubation in rat anti-Cx37 (1:100; Alpha Diagnostic, San Antonio, TX, USA) and mouse anti-Cx40 (1:100; Chemicon, Billerica, MA, USA), mouse anti-Cx43 (1:100; Chemicon) and mouse anti-Cx45 (1:100; Alpha Diagnostic) in a humidified chamber for 1 h. Endothelial cells were detected using polyclonal anti-factor VIII (1:500; Biocare Medical, Concord, CA, USA). Tissues were rinsed sequentially with normal PBS for 10 min, and then incubated in the secondary antibody (Vector, Burlingame, CA, USA). The slide was rinsed once again before adding the avidine–biotinylated horseradish peroxidase complex for 30 min (ABC/HRP, Vector). The complex was stained with diaminobenzidine for 2 min and the slide was counterstained with Mayer's haematoxylin for 30 s. All slides were compared with controls in which the primary antibody had been omitted. Microscopy was performed with an Axioplan 2 upright microscope with image capture by AXIOVISION software (Carl Zeiss, Oberkochen, Germany).

Immunostaining was evaluated in endothelial and muscle cells. The intensity is designated as 0 when no positive cells stain, 1 when 10–20% of cells stain (weak), 2 when 20–50% of cells stain (moderate) and 3 when 50% of cells stain (strong).

Data analysis

Data were expressed as mean ± SE. Vasorelaxant responses were expressed as percent inhibition of the contraction induced by PE. Concentration–response curves to ACh derived from each vessel were fitted separately to a logistic function by nonlinear regression and EC_{50} (molar concentration of ACh causing 50% of the maximal vasorelaxant effect) was calculated and expressed as log [M] (pEC_{50}). From the same regression, the maximum relaxation (R_{max}) was also calculated. Two-way ANOVA was used to compare concentration–response curves from controls and treated groups. Other data were analysed by one-way ANOVA or Student's *t*-test for paired or unpaired observations when appropriate. The *n* values quoted in parentheses indicate the number of experiments. The null hypothesis was rejected at $P < 0.05$.

Results

All rats treated with carbon tetrachloride included in the study had macronodular or micronodular cirrhosis. At the time of the study, no difference in the body weight between cirrhotic and control rats was observed.

Evaluation of the response to ACh of small mesenteric arteries preconstricted with PE

After treatment with indo, L-NAME and ODQ, the mesenteric arteries of cirrhotic rats ($n = 27$) showed a higher sensitivity to ACh in respect of control rats ($n = 26$) (two-way ANOVA, $P = 0.002$; R_{max}: 80 ± 4 vs. $70 \pm 5\%$, *P*: NS; EC_{50}: -6.55 ± 0.10 vs. -6.01 ± 0.10 log[M], $P = 0.0003$) (Fig. 1). Therefore, endothelium-dependent vasodilation is more prominent in the mesenteric arteries of cirrhotic rats in respect of control rats even after inhibiting COX, NOS and sGC.

Inhibition of epoxygenase with miconazole (inhibitor of EET production) did not modify the response to ACh in control rats ($n = 8$) (two-way ANOVA, *P*: NS; R_{max}: from 70 ± 5 to $69 \pm 15\%$, *P*: NS; EC_{50}: from -6.01 ± 0.10 to -6.16 ± 0.13 log[M], *P*: NS) (Fig. 2), while it significantly decreased maximal dilation in cirrhotic rats ($n = 9$) (two-way ANOVA, $P = 0.009$; R_{max}: from 80 ± 4 to $44 \pm 13\%$, $P = 0.0005$; EC_{50}: from -6.55 ± 0.10 to -6.37 ± 0.15 log[M], *P*: NS) (Fig. 2). Therefore, EETs seem to play a role in endothelium-dependent relaxation in cirrhotic rats but not in control rats.

18α-Glycyrrhetinic acid (a GJ blocker) reduced ACh-induced vasodilation both in control ($n = 5$) and in cirrhotic rats ($n = 8$) (two-way ANOVA, $P = 0.005$ and $P < 0.0001$ respectively), but while in control rats, it only decreased maximal dilation (R_{max}: from 70 ± 5 to $24 \pm 7\%$, $P = 0.0006$; EC_{50}: from -6.01 ± 0.10 to -6.28 ± 0.10

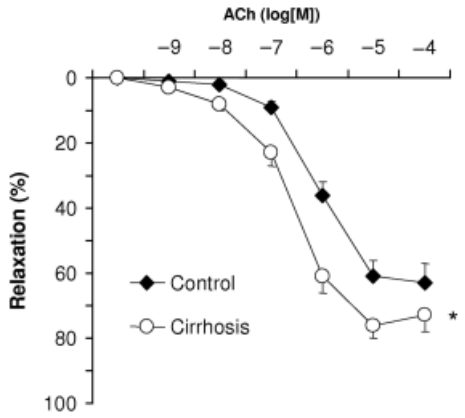

Fig. 1. Concentration–response curves to ACh obtained in small resistance mesenteric arteries incubated with indo (2.8 μM), L-NAME (1 mM) and ODQ (10 μM) to inhibit COX, NOS and sGC respectively. The effect of ACh was increased in cirrhotic rats with respect to control rats. *Significantly different ($P = 0.002$) from control (two-way ANOVA). ACh, acetylcholine; ANOVA, analysis of variance; COX, cyclo-oxygenase; L-NAME, N^G-nitro-L-arginine-methyl-ester; NOS, nitric oxide synthase; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; sGC, soluble guanylyl cyclase.

log[M], *P*: NS), in cirrhotic rats, it decreased both maximal dilation and sensitivity of the artery to ACh (R_{max}: from 80 ± 4 to $35 \pm 8\%$, $P < 0.0001$; EC_{50}: from -6.55 ± 0.10 to -6.07 ± 0.16 log[M], $P = 0.02$) (Fig. 2).

Evaluation of the response to 11,12-epoxyeicosatrienoic acid of small mesenteric arteries preconstricted with phenylephrine

Mesenteric arteries of cirrhotic rats ($n = 9$) showed a higher vasodilating response to 11,12-EET in respect of control rats ($n = 18$) (two-way ANOVA, $P = 0.002$) (Fig. 3). In control rats, the vasodilating effect of EET was totally inhibited by blocking BK_{Ca} channels with IbTx ($n = 5$) (two-way ANOVA, $P = 0.02$), but it was not modified by blocking GJs with 18α-GA ($n = 9$) (two-way ANOVA, *P*: NS); it was not reduced after the removal of endothelium ($n = 5$) (two-way ANOVA, *P*: NS) (Fig. 4). On the contrary, in cirrhotic rats, EET action was significantly reduced by 18α-GA (GJ inhibitor) ($n = 7$) (two-way ANOVA, $P = 0.003$) and only partially by IbTx (BK_{Ca} inhibitor) ($n = 5$) (two-way ANOVA, $P = 0.06$); it was markedly reduced after the removal of endothelium ($n = 7$) (two-way ANOVA, $P < 0.001$) (Fig. 4).

mRNA expression of connexins in mesenteric arteries

The mRNA expression of Cxs was evaluated by real-time reverse transcription-polymerase chain reaction in three control rats and in three cirrhotic rats. The expression of Cx40 and Cx43 mRNA was enhanced in cirrhotic rats in respect of control rats (Cx40: 4.145 ± 0.141 vs. 1.011 ± 0.109, $P < 0.001$; Cx43: 3.069 ± 0.171 vs 1.003 ± 0.050, $P < 0.001$). Cx37 and Cx45 mRNA expression was not different between control and cirrhotic rats (Fig. 5).

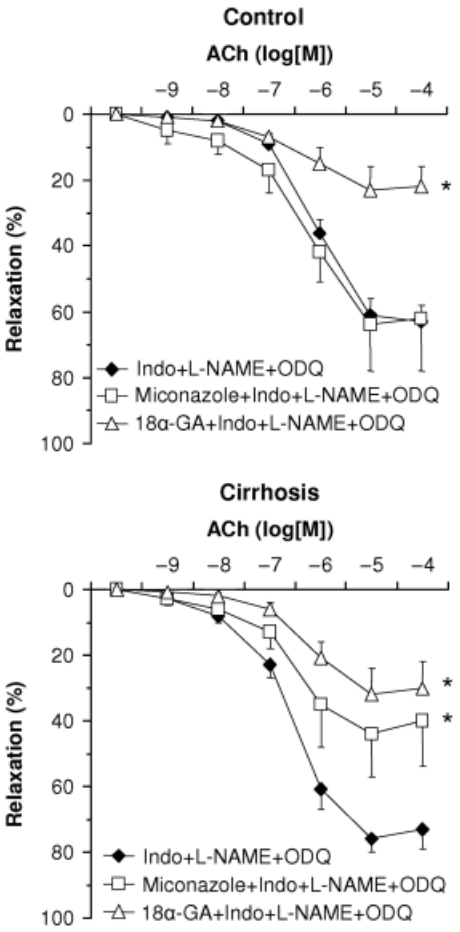

Fig. 2. Concentration–response curves to ACh obtained in small resistance mesenteric arteries incubated with indo (2.8 μM), L-NAME (1 mM) and 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (10 μM) to inhibit COX, NOS and sGC respectively. With respect to vehicle (closed diamond), incubation with the epoxygenase inhibitor miconazole (2 μM) (open square) decreased the effect of ACh in cirrhotic rats (two-way ANOVA, $P = 0.009$) but not in control rats (two-way ANOVA, *P*: NS). On the other hand, incubation with the GJs inhibitor 18α-GA (100 μM) (open triangle) decreased the effect of ACh both in control rats (two-way ANOVA, $P = 0.005$) and in rats with cirrhosis (two-way ANOVA, $P < 0.0001$), but in the latter group, the effect was more pronounced with a significant increase in EC_{50} (see text). *Significantly different ($P < 0.01$) from indo+L-NAME+ODQ (two-way ANOVA). ACh, acetylcholine; ANOVA, analysis of variance; COX, cyclo-oxygenase; indo, indomethacin; 18α-GA, 18α-glycyrrhetinic acid; GJ, gap junction; L-NAME, N^G-nitro-L-arginine-methyl-ester; NOS, nitric oxide synthase; ODQ, 1H-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one; sGC, soluble guanylyl cyclase.

Immunohistochemistry

Protein expression of Cxs was evaluated by immunohistochemistry in four control rats and in eight cirrhotic rats. In cirrhotic animals, endothelial cells showed a

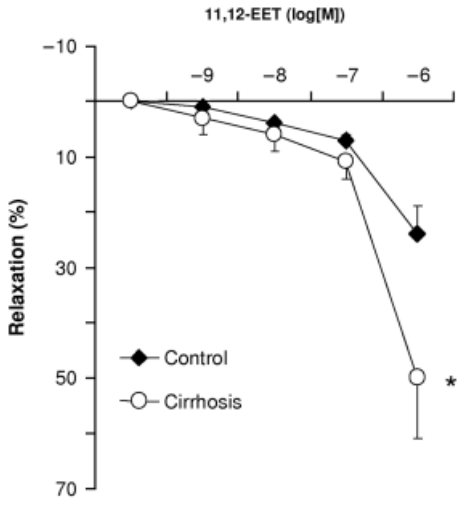

Fig. 3. Concentration–response curves to 11,12-EET in small resistance mesenteric arteries. The effect of 11,12-EET was increased in cirrhotic rats with respect to control rats. *Significantly different ($P = 0.002$) from control (two-way ANOVA). ANOVA, analysis of variance; EET, epoxyeicosatrienoic acid.

higher score of Cx40 and Cx43 compared with controls (Cx40: 2.75 ± 0.17 vs. 1.00 ± 0.87, $P = 0.02$; Cx43: 2.50 ± 0.19 vs 1.25 ± 0.75, $P = 0.05$). No differences were detected for Cx37 and Cx45. No significant differences were observed in muscle cells for all Cxs (Fig. 6).

Discussion

This study demonstrates that in small resistance mesenteric arteries of rats with CCl_4-induced cirrhosis, 11,12-EET and GJs participate in the increased mesenteric vasodilation characteristic of this pathological condition.

Endothelium-dependent vasodilation of mesenteric arteries was increased in cirrhotic rats (9), and it remained higher even after inhibiting NOS, COX and sGC. Therefore, systems other than NO, PGI_2 and cGC are involved in the abnormal mesenteric vasodilation of cirrhosis. The haeme oxygenase/carbon monoxide system is involved in this alteration (5, 8), but, according to the present study, EETs and GJs play a pivotal role.

After NOS, COX and sGC inhibition, miconazole did not modify the response to ACh in control mesenteric arteries, confirming that cytochrome P450 products do not have a significant role in the endothelium-dependent vasodilation of normal mesenteric arteries (26, 33). On the contrary, the inhibition of EET production by miconazole decreased the NO- and PGI_2-independent vasodilating effect of ACh in cirrhotic animals. Hence, EETs seem to play a role in endothelium-dependent relaxation in cirrhotic rats but not in control rats.

An EET-dependent component of endothelium-dependent relaxation appears to characterize cirrhosis. Thus, we studied the effect of EETs on mesenteric arteries. Vasodilation to EETs has been demonstrated in renal, mesenteric, cerebral, pulmonary and coronary

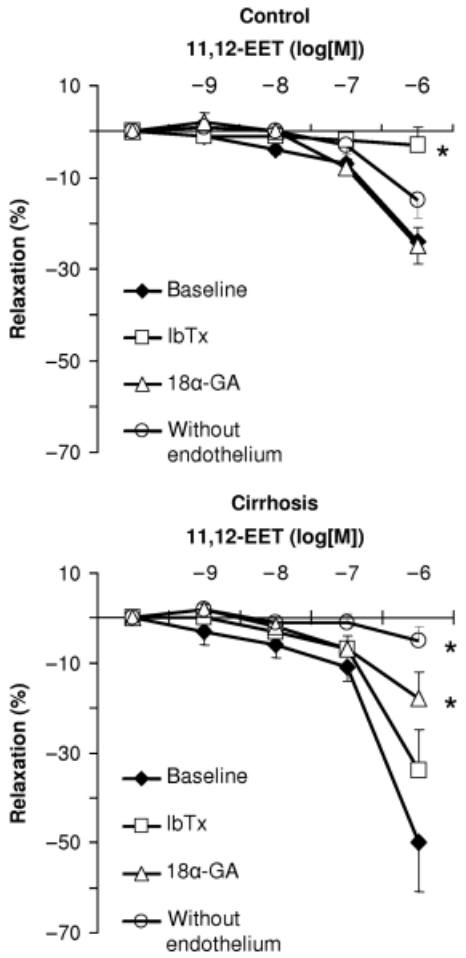

Fig. 4. Concentration–response curves to 11,12-EET obtained in small resistance mesenteric arteries. With respect to vehicle (closed diamond), incubation with the BK_{Ca} inhibitor IbTx (25 nM) (open square) decreased the effect of 11,12-EET in control rats (two-way ANOVA, $P = 0.02$) but not in cirrhotic rats (two-way ANOVA, $P = 0.06$). On the other hand, incubation with the GJs inhibitor 18α-GA (100 μM) (open triangle) decreased the effect of 11,12-EET in cirrhotic rats (two-way ANOVA, $P = 0.03$) but not in control rats (two-way ANOVA, *P*: NS). Removal of the endothelium (open circle) reduced the vasodilating response to 11,12-EET in cirrhotic rats (two-way ANOVA, $P < 0.001$) but not in control rats (two-way ANOVA, *P*: NS). *Significantly different ($P < 0.05$) from baseline (two-way ANOVA). 18α-GA; 18α-glycyrrhetinic acid; ANOVA, analysis of variance; EET, epoxyeicosatrienoic acid; GJ, gap junction.

arteries (14). Four EET regioisomers exist, 5,6-EET, 8,9-EET, 11,12-EET and 14,15-EET, which have different vasorelaxing properties depending on the specific organs investigated. In rat small mesenteric arteries, 11,12-EET is the most potent vasodilator among the four regioisomers (14). Moreover, 11,12-EET and 14,15-EET are the two predominant diffusible isoforms (19), and 11,12-EET is an EDHF in human internal mammary artery (13). In this study, taking together these data, we chose to evaluate the effect of the 11,12-isoform of EET.

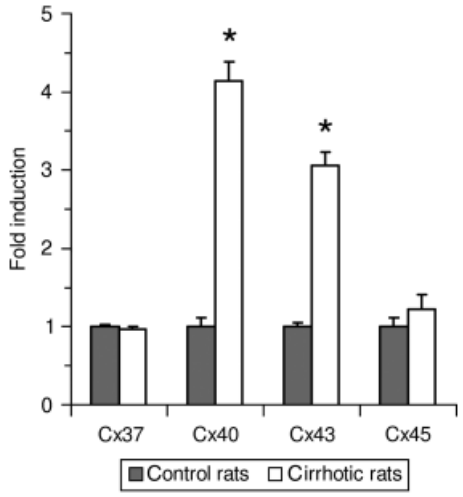

Fig. 5. Connexin mRNA expression in mesenteric arteries. mRNA transcripts were assessed in each sample of mesenteric arteries by real-time PCR using primers specific for rat Cxs genes. Changes in mRNA gene expression were reported as fold changes relative to controls (controls rats) using the $2^{-\Delta\Delta C_T}$ method (32). The results were normalized to the housekeeping gene. Results are representative of three independent experiments. $^*P < 0.001$ compared with control rats of the corresponding Cx group. Cx, connexin; PCR, polymerase chain reaction.

Our results showed that 11,12-EET vasodilates more the mesenteric arteries of cirrhotic rats than those of control rats. In control rats, the effect of 11,12-EET was modest, and was blocked by pretreatment with IbTx, but not with 18α-GA, confirming that in control rats, 11,12-EET primarily acts through the opening of BK_{Ca} (10–14) localized on VSMC. On the contrary, in cirrhotic rats, the effect of 11,12-EET was increased and was only slightly reduced by pretreatment with IbTx, but it was significantly inhibited by pretreatment with 18α-GA. Therefore, in cirrhotic rats, the increased vasodilating effect of 11,12-EET is only partially because of its effect on BK_{Ca}, even though these channels are hyperexpressed in cirrhosis (9). The increased effect of 11,12-EET is probably mediated by GJs, because it was blocked by 18α-GA. Therefore, in the mesenteric arteries of cirrhotic rats, 11,12-EET may act via the endothelium by initiating a hyperpolarizing response that is conducted to VSMC by myoendothelial GJ with consequent vasorelaxation. Indeed, the increased vasodilatory effect of 11,12-EET in cirrhotic rats was blunted after the removal of endothelium (Fig. 4).

The importance of GJs in mesenteric vasodilation of cirrhotic rats is highlighted by the analysis of the effect of 18α-GA on ACh-induced vascular dilation. The NOS/COX-independent component of endothelium-dependent vascular relaxation was inhibited more in cirrhotic than that in control rats by blocking GJs.

Gap junctions are protein channels connecting the cytoplasm of neighbouring cells, thus offering a low-resistance pathway for the passage of current and small molecules such as second messengers between cells (29). GJs consist of protein hemichannels, one from each cell, so-called Cxs, and each hemichannel is composed of six transmembrane proteins, Cxs (29), arranged around an aqueous central pore (34). The Cx37, Cx40, Cx43 and Cx45 are the predominant isoforms of GJ proteins expressed in the vascular wall (15, 29–31). They are abundant in the endothelial monolayer and sparse in the media (15).

The endothelial monolayer behaves as a conductive tissue propagating an electrical signal along the axis of the blood vessel by means of homocellular GJ and throughout the vascular wall itself by means of myoendothelial GJ (19). The spread of endothelial hyperpolarization through GJs is crucial to coordinate vascular relaxation along the vessel wall (35). Cxs play a critical role in EDHF-mediated dilation of rat mesenteric arteries (36). Cx37, Cx40 and Cx43 are involved in endothelial homocellular GJ (19). Cx37 and Cx40 are also involved in heterocellular GJ linking endothelial cells to VSMCs (19). A deficiency in Cx40 impairs longitudinal conduction of vasodilation and Cx40-deficient mice are hypertensive (30).

In our study, a significant increase of Cx40 and Cx43 in the mesenteric arteries of cirrhotic rats was documented both at the mRNA level and by its increased protein expression in the endothelium, demonstrated by immunohistochemistry.

An altered expression of Cxs has already been demonstrated in diseases associated with vascular complications, such as hypertension and diabetes (31). Indeed, in endothelial cells of the mesenteric artery of spontaneously hypertensive rats, there was a significantly lower expression of Cx37 and Cx40, which is corrected by the renin–angiotensin system blockade (37). The decreased expression of these Cxs may be involved in the altered vascular reactivity of spontaneously hypertensive rats (37). A reduced expression of Cxs40 and Cx43 in the endothelium of mesenteric arteries from spontaneously hypertensive rats has also been reported by Ellis *et al.* (38). In diabetes, the disruption of Cx43-mediated GJ communication induced by hyperglycaemia may contribute to the vascular dysfunction typically observed in this disease (31). On the other hand, an increased GJ communication, possibly because of increased GJ protein expression, may facilitate the effect of EDHFs, contributing to the augmented endothelium-dependent relaxation in arteries from pregnant rats (39). Moreover, in the mesenteric artery of female rats, oestrogen deficiency because of ovariectomy impairs EDHF-mediated vasodilation, because of a reduced expression of Cx43 (40), a result confirmed by Nawate *et al.* (41), who suggested that oestrogen deficiency following ovariectomy downregulates Cx40 and Cx43 proteins in rats, and that this may lead to a reduction of EDHF-mediated responses.

It can be hypothesized that in cirrhotic rats, the hyperpolarization of the endothelial cells is induced by the generation of EETs (22). The endothelial hyperpolarization could

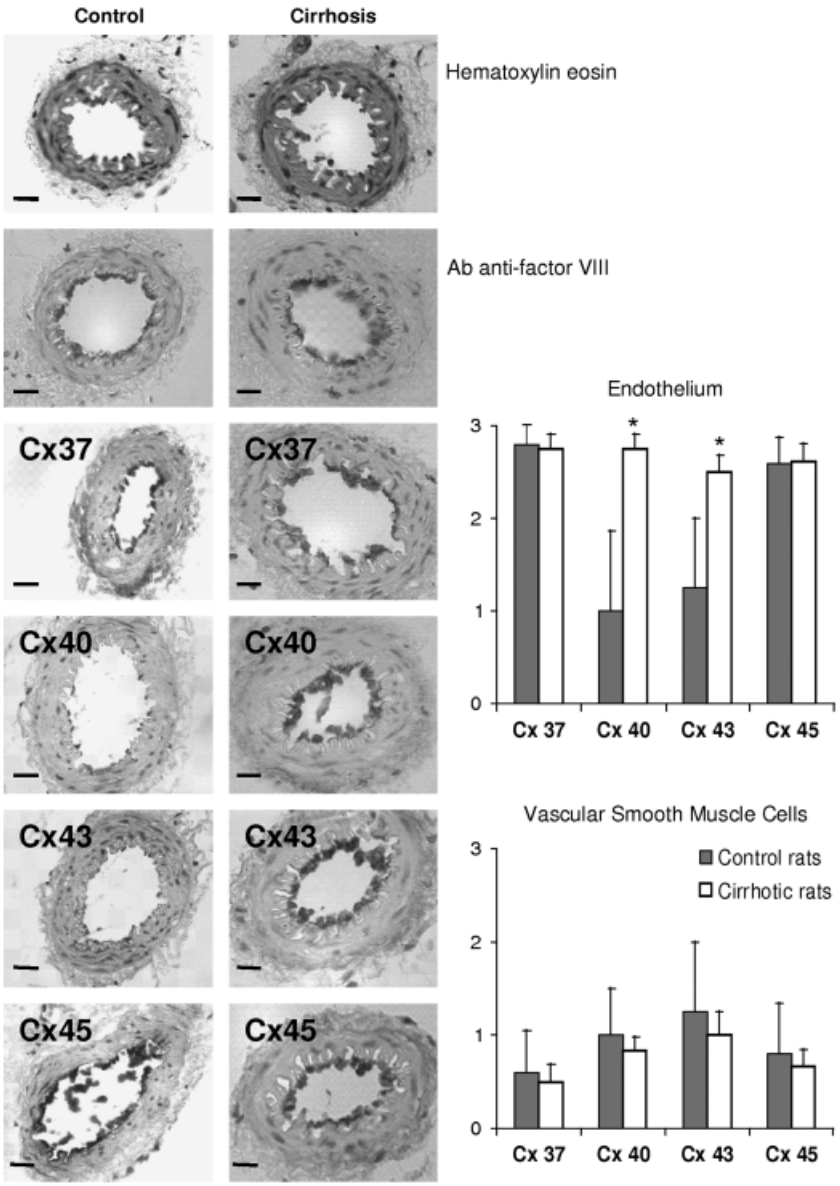

Fig. 6. Connexin (Cx) expression in resistance mesenteric arteries. Immunohistochemistry using antibodies against Cx37, Cx40, Cx43 and Cx45 showed an increased expression of Cx40 and Cx43 in endothelial cells in the mesenteric arteries of cirrhotic rats in respect to control rats. Immunostaining was quantified 0–3 according to the percentage of cell staining. The images are representative of four control rats and eight cirrhotic rats. $^{*}P < 0.05$ compared with the control rats of the corresponding Cx group. Scale bar: 50 μm.

then spread to the adjacent VSMCs through myoendothelial GJ, which might also be modulated by EETs (42, 43). Therefore, the increased expression of GJ may be responsible for the mesenteric vasodilation of cirrhotic rats or at least for the NO-/PGI_2-independent VSMC relaxation.

We do not know why only Cx40 and Cx43 are upregulated in cirrhosis. We can generate a hypothesis. There is enough strong evidence that Cx43 is upregulated by angiotensin II (44, 45) and by mechanical stress (shear stress) (31, 46–48). Therefore, the increased expression of Cx43 in the arteries of cirrhotic rats might be explained by the activation of the renin–angiotensin system, a common feature of cirrhosis (49), and by vascular shear stress, which may be increased during mesenteric vasodilation of cirrhotic rats.

The increased expression of Cx40 is more difficult to explain. We may hypothesize a role of the vascular overproduction of NO, which is one of the hallmarks involved in the pathogenesis of vasodilation in cirrhosis (1). Even though data on the relationships between NO

and GJ are not univocal (50, 51), we cannot rule out a link between a high level of NO and the increased expression of Cx40 in mesenteric vessels of cirrhotic rats.

In conclusion, the increased NO-/PGI_2-independent vasodilatation of mesenteric arterial circulation in cirrhosis is due, at least in part, to hyperreactivity to 11,12-EET through an increased expression of myoendothelial GJs.

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