

Research Paper

Cirrhosis decreases vasoconstrictor response to electrical field stimulation in rat mesenteric artery: role of calcitonin gene-related peptide

Javier Blanco-Rivero¹, Iván Márquez-Rodas², Esther Sastre¹, Ángel Cogolludo³, Francisco Pérez-Vizcaíno³, Lara del Campo¹, M^a Paz Nava⁴ and Gloria Balfagón¹

¹Departamento de Fisiología, Facultad de Medicina, Universidad Autónoma de Madrid, España

²Servicio de Oncología Médica, Hospital General Universitario Gregorio Marañón, Madrid, España

³Departamento de Farmacología and ⁴Departamento de Fisiología, Facultad de Medicina, Universidad Complutense de Madrid, Madrid, España

Our study determines alterations in the vasoconstrictor response elicited by electric field stimulation (EFS) in mesenteric arteries from cirrhotic rats treated with CCl₄, and how calcitonin gene-related peptide (CGRP) participates in this response. Vasoconstriction induced by EFS was analysed in the absence and presence of the CGRP receptor antagonist CGRP(8–37) in arterial segments from control and cirrhotic rats. The vasodilator response to exogenous CGRP was tested in both groups of rats, and the interference of the guanylate cyclase inhibitor ODQ or the K_{ATP} channel blocker glibenclamide was analysed only in segments from cirrhotic rats. The vasodilator response to the K_{ATP} channel opener pinacidil and to 8-bromo-cyclic GMP was tested. The K_{ATP} currents were recorded using the patch-clamp technique. Expression of receptor activity-modifying protein 1 (RAMP1), calcitonin receptor-like receptor, Kir 6.1 and sulfonylurea receptor 2B (SUR2B) was also analysed. Release of CGRP and cGMP was measured. The EFS-elicited vasoconstriction was less in segments from cirrhotic rats. The presence of CGRP(8–37) increased the EFS-induced response only in segments from cirrhotic rats. The CGRP-induced vasodilatation was greater in segments from cirrhotic rats, and was inhibited by ODQ or glibenclamide. Both pinacidil and 8-bromo-cyclic GMP induced a stronger vasodilator response in segments from cirrhotic rats. Pinacidil induced greater K_{ATP} currents in cirrhotic myocytes. Expression of RAMP1, calcitonin receptor-like receptor, Kir 6.1 and SUR2B was not modified by liver cirrhosis. Liver cirrhosis increased CGRP release, but did not modify cGMP formation. The decreased vasoconstrictor response to EFS in cirrhosis is mediated by increased vasodilator response to CGRP, as well as increased K_{ATP} channel gating. This effect of CGRP may play a role in the splanchnic vasodilatation present in liver cirrhosis.

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Corresponding author G. Balfagón: Departamento de Fisiología, Facultad de Medicina, Universidad Autónoma de Madrid, C/Arzobispo Morcillo 4, 28029 Madrid, Spain. Email: gloria.balfagon@uam.es

Liver cirrhosis is ranked as one of the 10 most common causes of death in the Western world (Stewart & Day, 2003) and is associated, among other alterations, with portal hypertension and hyperdynamic circulation (Henriksen *et al.* 1999), in which peripheral arterial vasodilatation, mainly in the splanchnic circulation, plays a major role (Schrier *et al.* 1988). This decrease of vascular resistance is associated with a modification of endothelial factors in these vessels, including increased release and sensitivity

to vasodilator factors such as nitric oxide (NO; Morales-Ruiz *et al.* 1996; Iwakiri, 2007) and prostaglandin I₂ (Vaughan *et al.* 2005), as well as a decreased response to vasoconstrictor factors such as noradrenaline (NA; Battaglia *et al.* 2006), phenylephrine (Van Obbergh *et al.* 1995; Ebrahimkhani *et al.* 2008) or U-46619 (Van Obbergh *et al.* 1995).

Vascular tone is determined by a balance of several mechanisms, in which innervation plays an important role

that varies in the different vascular beds. Adrenergic (Li & Duckles, 1992) and nitrergic innervations (Yu *et al.* 1993; Marín & Balfagón, 1998) have been described in the rat mesenteric bed, respectively releasing NA and NO on electrical stimulation. Previous reports show alterations in adrenergic innervation (Coll *et al.* 2008, 2010), decreased NA release (Kirstetter *et al.* 1998; Gatta *et al.* 2008) and increased neuronal NO synthase levels (Gatta *et al.* 2008) in cirrhosis, implicating these mechanisms in the hyperdynamic circulation present in liver cirrhosis. Additionally, rat mesenteric arteries possess rich sensory innervation (Kawasaki *et al.* 1988); when electrically stimulated, the innervation triggers the release of the vasodilator neurotransmitter calcitonin gene-related peptide (CGRP), which activates specific receptors on smooth muscle cells (Márquez-Rodas *et al.* 2006b). The CGRP receptor is one of the superfamily of G-protein-coupled receptors (GPCRs) and is composed of three subunits: a seven-transmembrane-domain G-protein-coupled receptor called calcitonin receptor-like receptor (CL receptor), the single-transmembrane-spanning receptor activity-modifying protein 1 (RAMP1; Poyner *et al.* 2002) and the receptor component protein (RCP; Brain & Grant, 2004). The expression of RAMP1 in combination with CL receptor produces a specific and functional CGRP receptor. An increase in vasodilator response to CGRP has been related to an enhanced expression of RAMP1 subunit in rat mesenteric artery (Márquez-Rodas *et al.* 2006a), while changes in CL receptor expression without an increase in RAMP1 do not correlate with changes in the functional role of CGRP receptor (Márquez-Rodas *et al.* 2008).

It has been previously reported that the vasodilator response to CGRP in rat mesenteric artery depends on activation of ATP-dependent potassium (K_{ATP}) channels, calcium-dependent potassium (K_{Ca}) channels, cAMP and cGMP (Wimalawansa, 1996; Balfagón *et al.* 2004).

In contrast, we have reported that different physiopathological situations, such as hypertension (Marín *et al.* 2000) and hyperaldosteronism (Balfagón *et al.* 2004), or sex steroids (del Campo *et al.* 2009) can modify the role of sensory innervation in vascular tone. However, as yet there have been no studies to analyse the possible implication of sensory innervation in the splanchnic vasodilatation observed in liver cirrhosis. Knowledge of the possible participation of CGRP in liver cirrhosis would open the possibility of therapeutic approaches to the splanchnic vasodilatation present in this pathology.

Therefore, the aim of this study was to determine whether liver cirrhosis alters the vasomotor response to electric field stimulation (EFS) in mesenteric arteries, and how sensory innervation, specifically through CGRP, may participate in this response. Another focus was to identify the possible mechanisms implicated in this process.

Methods

Animals

Male Sprague–Dawley rats (6 months old) were used. These were divided into two groups: control and cirrhotic rats. All animals were housed in the Animal Facility of the Universidad Autónoma de Madrid (registration number EX-021U) in accordance with directives 609/86 of the EEC and RD 233/88 of the Ministerio de Agricultura, Pesca y Alimentación of Spain, and the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996).

Cirrhosis was induced when rats were 4 months old by intragastric administration of carbon tetrachloride (CCl_4 ; 0.2 ml (100 g body weight)⁻¹, twice a week; diluted 1:4 in olive oil) along with phenobarbital in the drinking water (0.35 g l⁻¹), for 9 weeks, as previously described (Xu *et al.* 2000; Fang *et al.* 2007).

Systolic blood pressure was indirectly measured in awake animals by the tail-cuff method, as previously reported (Buñag, 1973; Digital Pressure Meter, LE5000, Letica, Barcelona, Spain). Rats were killed by CO₂ inhalation; the first branch of the mesenteric artery was carefully dissected out, cleaned of connective tissue and placed in Krebs–Henseleit solution (KHS, in mmol l⁻¹: NaCl, 115; CaCl₂, 2.5; KCl, 4.6; KH₂PO₄, 1.2; MgSO₄·7H₂O, 1.2; NaHCO₃, 25; glucose, 11.1; and Na₂EDTA, 0.03) at 4°C.

Measurement of portal pressure

Splenic pulp pressure, an effective indirect measurement of portal pressure, was measured by inserting a 20 gauge fluid-filled needle into the spleen parenchyma. The needle was joined to a PE-50 tube connected to a pressure recorder (PowerLab 200 ML 201) and a transducer (Sensoror SN-844) with a Chart V4.0 computer program (AD Instruments, Oxford, UK); the recorder was recalibrated before each experiment.

Mesenteric venous vasculopathy and portosystemic collateral circulation study

The existence of portal hyperpressure in cirrhotic rats was confirmed by the development of mesenteric venous vasculopathy and portosystemic collateral circulation. First, a mid-line abdominal incision was made with a large bilateral subcostal extension. Mesenteric venous vasculopathy, a characteristic feature of splanchnic venous congestion, was observed as dilatation and tortuosity of the superior mesenteric vein branches (Corcuera *et al.* 2005).

Portosystemic collateral circulation was studied by macroscopic examination of the areas in which the collateral venous circulation had developed (splenorenal, gastro-oesophageal, colorectal and hepatic hilum), carefully identifying the development of collateral veins (Diéguez *et al.* 2002).

Vascular reactivity

The method used for recording of isometric tension has been described in full elsewhere (Nielsen & Owman, 1971). Briefly, two parallel stainless-steel pins were introduced through the lumen of the vascular segment; one was fixed to the bath wall and the other connected to a force transducer (Grass FTO3C, Quincy, MA, USA); in turn, this was connected to a model 7D Grass polygraph. For EFS experiments, segments were mounted between two platinum electrodes 0.5 cm apart and connected to a stimulator (Grass, model S44) modified to supply the appropriate current strength. Segments were suspended in an organ bath containing 5 ml of KHS at 37°C continuously bubbled with a 95% O₂–5% CO₂ mixture (pH 7.4). Experiments were performed in endothelium-denuded segments to eliminate the main source of vasoactive substances, including endothelial NO. This avoided possible actions by different drugs on endothelial cells that could lead to misinterpretation of results. Endothelium was removed by gently rubbing the luminal surface of the segments with a thin wooden stick. The segments were subjected to a tension of 4.9 mN, which was re-adjusted every 15 min during a 90 min equilibration period before drug administration. After this, the vessels were exposed to 75 mmol l⁻¹ KCl to check their functional integrity. Removal of the endothelium did not alter the contractions elicited by 75 mmol l⁻¹ KCl. After a washout period, the absence of vascular endothelium was tested by the inability of 10 μmol l⁻¹ acetylcholine (ACh) to relax segments precontracted with NA (control, 9.48 ± 0.9 mN; cirrhotic, 9.34 ± 1.02 mN).

Frequency–response curves to EFS (1, 2, 4, 8 and 16 Hz) were obtained in segments from control and cirrhotic rats. The parameters used for EFS were 200 mA, 0.3 ms, 1–16 Hz, for 30 s with an interval of 1 min between each stimulus, the time required to recover basal tone. A washout period of at least 1 h was necessary to avoid desensitization between consecutive curves. Three successive frequency–response curves separated by 1 h intervals produced similar contractile responses. When assessing the effect of tetrodotoxin, a blocker for nerve impulse propagation (TTX, 0.1 μmol l⁻¹), on the contraction elicited by EFS, the substance was added to the bath 30 min in advance.

To analyse the possible participation of sensory innervation in the EFS-induced response in control and

cirrhotic rats, 0.5 μmol l⁻¹ CGRP(8–37), a CGRP receptor antagonist, was added to the bath 30 min before obtaining the second frequency–response curve.

The vasoconstrictor response induced by exogenous NA (1 nmol l⁻¹ to 10 μmol l⁻¹) was tested in segments from control and cirrhotic animals. The vasodilator effect of exogenous CGRP (0.1 nmol l⁻¹ to 1 μmol l⁻¹) was tested in NA-precontracted arteries from control and cirrhotic animals. The different intracellular signalling pathways produced by CGRP were studied only in mesenteric segments from cirrhotic rats by obtaining concentration–response curves for exogenous CGRP in the presence of the protein kinase A selective inhibitor RP-cyclic Adenosine monophosphate (RP-cAMP; 5 μmol l⁻¹), the guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10 μmol l⁻¹), the K_{ATP} channel blocker glibenclamide (10 μmol l⁻¹), or a combination of the K_{Ca} channel blockers charybdotoxin (0.1 μmol l⁻¹) and apamin (1 μmol l⁻¹).

To analyse the vasodilator response to the K_{ATP} channel opener pinacidil, concentration–response curves (1 nmol l⁻¹ to 10 μmol l⁻¹) were obtained in segments from both groups of rats in NA-precontracted segments. At the end of the experiment, arterial rings were exposed to papaverine (0.1 mmol l⁻¹) to determine the maximal relaxant response of the tissue. To study the participation of specific K_{ATP} channels in this vasodilator response, glibenclamide (10 μmol l⁻¹) was added 30 min before the concentration–response curves were obtained.

The vasodilator effect of 8-bromo-cyclic GMP (8Br-cGMP, 0.1 μmol l⁻¹ to 0.1 mmol l⁻¹) was tested in NA-precontracted arteries from control and cirrhotic animals.

Patch clamp

For myocyte isolation, endothelium-denuded mesenteric arteries from both groups were cut into small segments and placed into a nominally Ca²⁺-free physiological salt solution (PSS) of the following composition (in mmol l⁻¹): NaCl, 130; KCl, 5; MgCl₂, 1.2; glucose, 10; and Hepes, 10 (pH adjusted to 7.3 with NaOH) containing (in mg ml⁻¹): papain, 1; dithiothreitol, 0.8; and albumin, 0.7 for 15 min. Thereafter, arterial segments were incubated for an additional 5 min in Ca²⁺-free PSS containing (in mg ml⁻¹): collagenase F, 1; collagenase H, 0.3; and albumin, 0.7. Cells were stored in Ca²⁺-free PSS (4°C) and used within 8 h of isolation. The K_{ATP} current was recorded using the whole-cell configuration of the patch-clamp technique as previously described (Cogolludo *et al.* 2007). In order to minimize interference with other K⁺ currents, the K_{ATP} current was measured at –80 mV. Cells were continuously held at this potential and initially perfused with Ca²⁺-free PSS; after 5 min, the superfusion medium was changed to a high-K⁺

(135 mmol l⁻¹) PSS (replacing NaCl with an equimolar concentration of KCl; pH adjusted to 7.4 with KOH). The internal patch pipette solution contained (in mmol l⁻¹): KCl, 130; Hepes, 10; MgCl₂, 1.2; MgATP, 1; and EGTA, 10 (pH adjusted to 7.3 with KOH). To analyse the K_{ATP} current, cells were superfused with the potassium channel opener pinacidil (1 μmol l⁻¹) and, once a stable response was achieved, subsequently superfused with glibenclamide (10 μmol l⁻¹) in the continuous presence of pinacidil.

Release of CGRP

To measure the release of CGRP, we used a rat CGRP enzyme immunoassay kit (SPI-Bio, Bertin Pharma, Montigny le Bretonneux, France). Briefly, endothelium-denuded segments of rat mesenteric arteries from control and cirrhotic rats were pre-incubated for 30 min in 5 ml of KHS at 37°C and continuously gassed with a 95% O₂–5% CO₂ mixture (stabilization period). This was followed by two washout periods of 10 min in a 0.4 ml bath containing KHS, after which arteries were subjected to cumulative EFS periods of 30 s at 1, 2, 4, 8 and 16 Hz at 1 min intervals. The different assays were performed according to the manufacturer's instructions. Results are expressed as picograms of CGRP per millilitre per milligram of tissue.

Determination of cGMP

Denuded rat mesenteric arteries from control and cirrhotic animals were subjected to a resting tension of 4.9 mN, as indicated in the reactivity experiments. After an equilibration period of 60 min, segments were contracted with NA for 3 min (considered the basal level), and then some segments were incubated with 0.1 μmol l⁻¹ CGRP for 10 s. Segments were immediately frozen in liquid nitrogen and stored at 70°C. Levels of cGMP were determined using the cGMP Direct Immunoassay Kit from Abcam (Cambridge, UK). For this assay, the frozen arteries were homogenized in 0.1 M HCl and centrifuged at 600g for 10 min at 4°C. The insoluble fraction was used to measure protein content with a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The supernatant was then collected and used for the assay. The cGMP levels were measured following the manufacturer's protocol. Results are expressed as picomoles of cGMP per milligram of protein.

Western blot analysis of RAMP1, CL receptor, Kir 6.1 and sulfonylurea receptor 2b (SUR2B)

For Western blot analysis, mesenteric segments from both groups of rats were homogenized in a boiling buffer composed of 1 mmol l⁻¹ sodium vanadate, 1% SDS and 0.01 mol l⁻¹ Tris–HCl (pH 7.4). Homogenates containing 30 μg protein were electrophoretically separated on

a 7.5% SDS–polyacrylamide gel for SUR2B, 10% SDS–polyacrylamide gel for Kir 6.1, or 12% SDS–polyacrylamide gel for RAMP1 and CL receptor, and then transferred to polyvinyl difluoride membranes (Bio-Rad Immun-Blot) overnight at 4°C, 230 mA, using a Bio-Rad Mini Protean III system (Bio-Rad) containing 25 mmol l⁻¹ Tris, 190 mM glycine, 20% methanol and 0.05% SDS. The membrane was blocked for 1 h at room temperature in Tris-buffered saline solution (100 mM, 0.9% w/v NaCl, 0.1% SDS) with 5% powdered fat-free milk before being incubated overnight at 4°C with goat polyclonal antibody against SUR2B (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal antibody against Kir 6.1 (1:500 dilution; Abcam, Cambridge, UK), rabbit polyclonal antibody against RAMP1 (1:250 dilution; Santa Cruz Biotechnology) or CL receptor (1:500 dilution; Abcam). After washing, the membrane was incubated with appropriate secondary horseradish peroxidase-conjugated immunoglobulin G (Amersham International Plc, Little Chalfont, UK). The membrane was thoroughly washed and the immunocomplexes were then detected using an enhanced horseradish peroxidase/luminol chemiluminescence system (ECL Plus; Amersham International Plc) and subjected to autoradiography (Hyperfilm ECL; Amersham International Plc). Signals on the immunoblot were quantified using a computer program (NIH Image version 1.56; National Institutes of Health, Bethesda, MD, USA). The same membrane was used to determine α-actin expression, and the content of the latter was used to correct protein expression in each sample by means of a monoclonal anti-α-actin antibody (1:2000 dilution; Sigma-Aldrich, Madrid, Spain). Rat brain homogenates were used as a positive control for RAMP1, CL receptor and SUR2B, and mouse pancreas as a positive control for Kir 6.1.

Drugs used

L-Noradrenaline hydrochloride, ACh chloride, CGRP(8–37), rat CGRP, TTX, L-NAME hydrochloride, glibenclamide, charybdotoxin, apamin, pinacidil, tetrodotoxin, ODQ and RP-cAMP were from Sigma-Aldrich. Stock solutions (10 mmol l⁻¹) of drugs were made in distilled water, except for NA, which was dissolved in a NaCl (0.9%) and ascorbic acid (0.01% w/v) solution, and glibenclamide and pinacidil, which were dissolved in ethanol. These solutions were kept at –20°C, and appropriate dilutions were made in KHS on the day of the experiment.

Data analysis

The responses elicited by EFS and exogenous NA were expressed as a percentage of the initial contraction elicited

Table 1. Effect of liver cirrhosis on body weight (BW), systemic blood pressure, portal pressure, spleen weight to body weight ratio (SW/BW) and liver weight to body weight ratio (LW/BW) in Sprague–Dawley rats

	BW (g)	Systemic blood pressure (mmHg)	Portal pressure (mmHg)	SW/BW (%)	LW/BW (%)
Control	525.5 ± 6.4	149.6 ± 4.1	6.36 ± 0.56	0.1 ± 0.008	3 ± 0.1
Cirrhotic	487.9 ± 6.9*	144.4 ± 3.4	8.42 ± 0.53*	0.2 ± 0.009*	4.1 ± 0.1*

Results are expressed as means ± S.E.M. * $P < 0.05$ versus control. $n = 10$ animals in each group.

by 75 mmol l⁻¹ KCl for comparison between control and cirrhotic rats. The relaxation induced by CGRP was expressed as a percentage of the initial contraction elicited by NA. Results are given as means ± S.E.M. Statistical analysis was done by comparing the curve obtained in the presence of the different substances with the previous or control curve by means of repeated-measure ANOVA with the Bonferroni *post hoc* test. For the experiments investigating CGRP release, cGMP determination and protein expression, the statistical analysis was done using one-way ANOVA with the Newman–Keuls multiple comparison test as a *post hoc* test. A value of $P < 0.05$ was considered significant.

Results

Animal parameters

Liver cirrhosis did not modify systemic blood pressure, but it did increase portal pressure (Table 1). Body weight was lower in cirrhotic animals, and all cirrhotic animals showed spleen hypertrophy and hepatomegaly (Table 1). Macro/micronodular cirrhosis, fibrosis and steatosis were present in cirrhotic livers. All rats in the liver cirrhosis group exhibited a portosystemic collateral circulation (pararectal, para-oesophageal, splenorenal and portohepatic collateral vessels).

Vascular reactivity

The response induced by 75 mmol l⁻¹ KCl was similar in segments from both groups of rats (control, 11.34 ± 0.6 mN; cirrhotic, 12.37 ± 1.1 mN; $P > 0.05$; $n = 10$ each group). The contractions induced by EFS were lower in mesenteric arterial segments from cirrhotic rats (Fig. 1A). The EFS-induced contractions were practically abolished in segments from both experimental groups by the blocker of nerve impulse propagation, TTX (0.1 μmol l⁻¹; Fig. 1B and C).

The CGRP receptor antagonist CGRP(8–37) (0.5 μmol l⁻¹) did not modify the contractile response induced by EFS in segments from control rats (Fig. 2A), but the response increased in segments from cirrhotic rats (Fig. 2B).

Vasoconstriction induced by exogenous NA (1 nmol l⁻¹ to 10 μmol l⁻¹) was less in mesenteric segments from cirrhotic rats than in those from control animals (Fig. 3A).

In segments precontracted with NA, CGRP (0.1 nmol l⁻¹ to 1 μmol l⁻¹) induced a concentration-dependent relaxation, which was significantly greater in segments from cirrhotic than from control rats (Fig. 3B). Pre-incubation of segments from cirrhotic rats with 5 μmol l⁻¹ RP-cAMP or with 0.1 μmol l⁻¹ charybdotoxin and 1 μmol l⁻¹ apamin did not modify the vasodilator response to CGRP, while pre-incubation with either 10 μmol l⁻¹ ODQ or 10 μmol l⁻¹ glibenclamide decreased it (Fig. 3B).

In NA-precontracted segments from control and cirrhotic rats, the K_{ATP} opener pinacidil (1 nmol l⁻¹ to 10 μmol l⁻¹) elicited a concentration-dependent relaxation, which was greater in cirrhotic than in control rats (Fig. 4A). The response elicited by pinacidil in the presence of the K_{ATP} blocker glibenclamide (10 μmol l⁻¹) was reduced to a greater extent in segments from cirrhotic rats (Fig. 4A). The vasodilator response to 0.1 mmol l⁻¹ papaverine was similar in both experimental groups (control, 15.12 ± 1.9 mN; cirrhotic, 14.99 ± 1.3 mN; $P > 0.05$), indicating that liver cirrhosis did not alter smooth muscle vasodilator capacity.

The vasodilator response to the non-hydrolysable analogue of cGMP, 8Br-cGMP (0.1 μM to 0.1 mM), was greater in segments from cirrhotic rats (Fig. 4B).

K_{ATP} currents

The average capacitance of the freshly isolated myocytes from control and cirrhotic arteries was 21.5 ± 0.61 ($n = 5$) and 20.1 ± 1.5 pF ($n = 6$), respectively ($P > 0.05$). Perfusion with 10 μmol l⁻¹ pinacidil increased an inward current in cells from control (Fig. 5A) and cirrhotic rats (Fig. 5B), and this was reverted by glibenclamide in both cell types. The magnitude of the glibenclamide-sensitive K_{ATP} current was much greater in smooth muscle cells from cirrhotic than control animals (Fig. 5C).

Release of CGRP

Basal CGRP release was greater in segments from cirrhotic than from control rats (Fig. 6A). Electric field stimulation increased CGRP levels in both groups of rats, but the

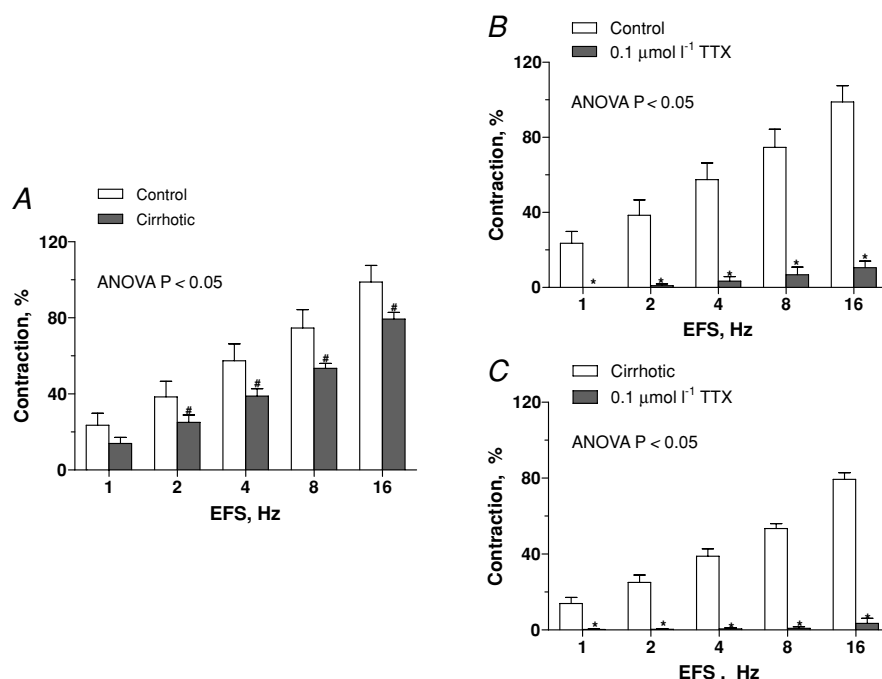


Figure 1. Vasoconstrictor response to electrical field stimulation in mesenteric segments from control and cirrhotic rats

A, isometric tension recording of the frequency-dependent contractions in denuded mesenteric artery segments from control and cirrhotic rats. Also shown is the effect of pre-incubation with $0.1 \mu\text{mol l}^{-1}$ TTX on the frequency-response curves obtained in mesenteric artery segments from control (B) and cirrhotic rats (C). Results (means \pm S.E.M.) are expressed as a percentage of tone induced by 75 mmol l^{-1} KCl. $n = 4-6$ in each group.

increase was greater in segments from cirrhotic rats (Fig. 6A).

Determination of cGMP

Basal cGMP levels were similar in segments from both groups (Fig. 6B). Incubation with $0.1 \mu\text{mol l}^{-1}$ CGRP for 10 s increased cGMP levels in segments from control and cirrhotic animals to a similar extent (Fig. 6B).

Expression of RAMP1, CL receptor, Kir 6.1 and SUR2B

RAMP1 was detected as a 30 kDa band on Western Blot. Expression of RAMP1 was not modified by cirrhosis (Fig. 7). Two bands of CL receptor with respective molecular weights of 110 and 66 kDa were detected, and they were not modified by liver cirrhosis (Fig. 7). Liver cirrhosis did not modify Kir 6.1 or SUR2B expression in mesenteric arterial segments (Fig. 7).

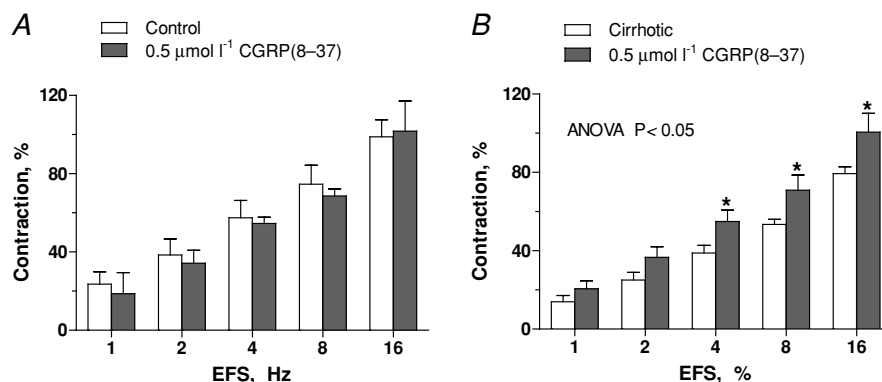


Figure 2. Effect of CGRP(8-37) on the frequency-response curves determined in mesenteric artery segments from control (A) and cirrhotic rats (B)

Results (means \pm S.E.M.) are expressed as a percentage of tone induced by 75 mmol l^{-1} KCl. $n = 4-6$ in each group. * $P < 0.05$ vs control situation.

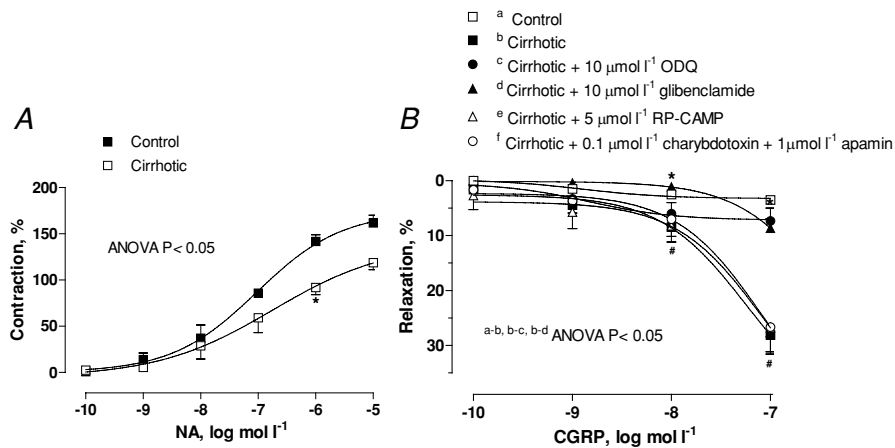


Figure 3. Vasomotor responses to NA and CGRP in mesenteric segments from control and cirrhotic rats
 A, vasoconstrictor response to NA in mesenteric artery segments from control and cirrhotic rats. Results (means \pm S.E.M.) are expressed as a percentage of tone induced by 75 mmol l⁻¹ KCl. $n = 4-6$ in each group. B, vasodilator response to CGRP in mesenteric artery segments from control and cirrhotic rats. Effect of 10 μ mol l⁻¹ ODQ, 10 μ mol l⁻¹ glibenclamide, 5 μ mol l⁻¹ RP-cAMP or 0.1 μ mol l⁻¹ charybdotoxin plus 1 μ mol l⁻¹ apamin on the vasodilator response to CGRP in segments from cirrhotic rats. Results (means \pm S.E.M.) are expressed as a percentage of the inhibition of the NA-induced contraction. $n = 4-6$ in each group.

Discussion

The results of the present study demonstrate for the first time that sensory innervation is implicated in the decreased vasoconstrictor EFS response in experimental liver cirrhosis, through an increased vasodilator response to CGRP, which is associated with an increase in K_{ATP} channel function.

Cirrhosis was induced with a 9 week CCl₄ treatment. All CCl₄-treated rats used in these experiments showed macroscopic macro/micronodular cirrhosis of the liver, hepatomegaly, splenic hypertrophy and increased portal pressure values. No rats developed ascites, something

which was described by Wiest (2007)) when CCl₄ administration was maintained for 12–20 weeks.

The vasoconstrictor response to KCl was similar in mesenteric segments from both experimental groups. These results concur with previous studies made in aortic segments from rats with ligated bile ducts (Hennenberg *et al.* 2006), but contrast with others which showed hyporeactivity to KCl in mesenteric segments from rats with biliary cirrhosis (Yang *et al.* 2007), indicating that different experimental models produce different effects.

Electric field stimulation induced contractile responses in endothelium-denuded mesenteric segments from control and cirrhotic rats. This response was diminished

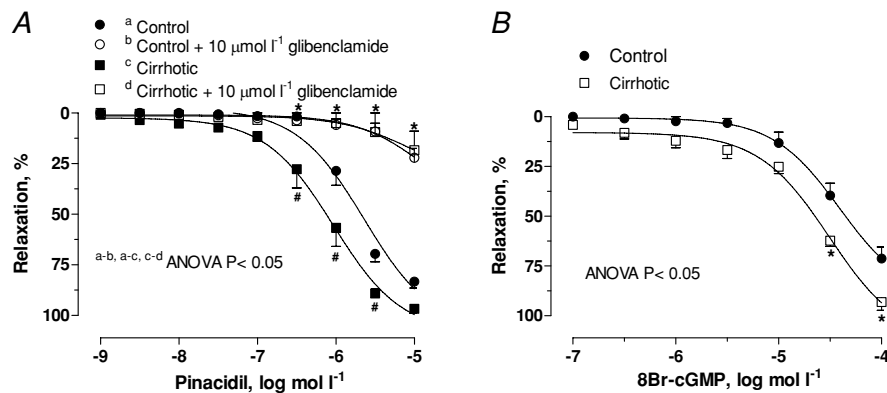


Figure 4. Vasodilator responses to pinacidil and 8Br-cGMP in mesenteric segments from control and cirrhotic animals
 A, effect of 10 μ mol l⁻¹ glibenclamide on the vasodilator response to pinacidil in mesenteric artery segments from control and cirrhotic rats. B, vasodilator response to 8Br-cGMP in segments from control and cirrhotic rats. Results (means \pm S.E.M.) are expressed as a percentage of the inhibition of the NA-induced contraction. $n = 4-6$ in each group.

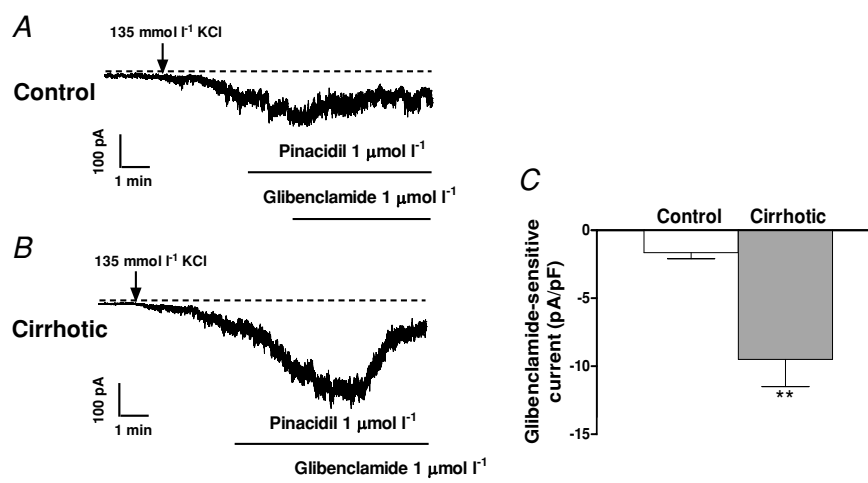


Figure 5. Different contributions by the K_{ATP} current in cells from control and cirrhotic rats

Effects of 1 μmol l⁻¹ pinacidil and their reversal by 1 μmol l⁻¹ glibenclamide on membrane currents in mesenteric artery smooth muscle cells isolated from control (A) and cirrhotic rats (B). Both panels show representative traces obtained in the whole-cell recording mode when cells were held at -80 mV. The dashed line represents zero current. C illustrates the average values (means ± s.e.m. of 5 experiments) of the glibenclamide-sensitive current at -80 mV in cells from control and cirrhotic rats. ** $P < 0.01$.

by cirrhosis. The vasoconstrictor response elicited by EFS was practically abolished by the blocker for nerve impulse propagation TTX, demonstrating that the EFS response was due to neurotransmission. Thus, the diminished vasoconstrictor response present in mesenteric segments from the cirrhotic group seems to be associated with changes in the release of and/or response to the different neurotransmitters released by EFS. Rat mesenteric arteries possess sympathetic (Li & Duckles, 1992), nitrergic (Yu *et al.* 1993; Marín & Balfagón, 1998) and sensory innervations (Kawasaki *et al.* 1988), all of which work together to modulate the vasomotor function. Previous studies have reported decreased NA release (Kirstetter *et al.* 1998; Gatta *et al.* 2008), downregulation of genes related

to the adrenergic system (Coll *et al.* 2008) and atrophy in the mesenteric sympathetic innervation (Coll *et al.* 2010). Our present results show a decreased vasoconstrictor response to exogenous NA in segments from cirrhotic animals. Additionally, increased neuronal NO synthase levels (Gatta *et al.* 2008) have been reported in cirrhosis. Therefore, all these mechanisms could be involved in the hyperdynamic circulation present in liver cirrhosis.

Since an increase in circulating CGRP levels has also been demonstrated in liver cirrhosis (Bendtsen *et al.* 1991; Henriksen *et al.* 1999), we studied the participation of the sensory neurotransmitter CGRP in the EFS-induced response in segments from both control and cirrhotic rats. Pre-incubation with the CGRP antagonist CGRP(8–37)

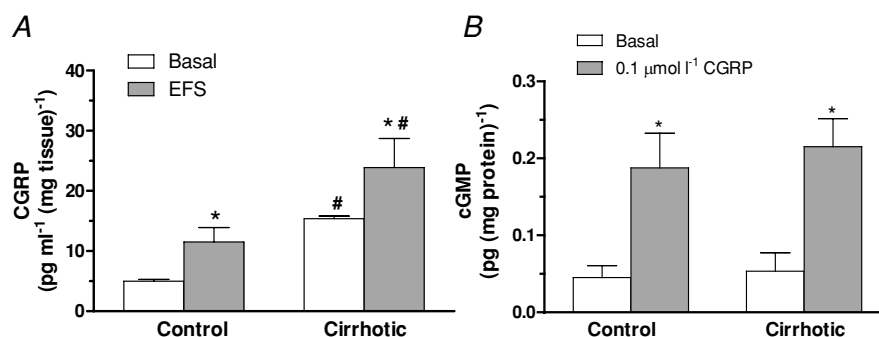


Figure 6. CGRP and cGMP releases in control and cirrhotic mesenteric segments

A, effect of liver cirrhosis on basal and EFS-induced CGRP release in mesenteric segments. Results are expressed as picograms of CGRP per millilitre per milligram of tissue. * $P < 0.05$ versus basal; # $P < 0.05$ versus control. $n = 4–6$ in each group. B, effect of liver cirrhosis on basal and CGRP-induced cGMP production in mesenteric segments. Results are expressed as picograms of CGRP per millilitre per milligram of tissue. * $P < 0.05$ versus basal. $n = 4–6$ in each group.

did not modify the vasoconstrictor response to EFS in mesenteric rings from control rats, indicating the non-participation of CGRP in the regulation of vascular tone in the healthy Sprague–Dawley rat mesenteric bed. However, CGRP(8–37) increased the vasoconstrictor response to EFS in segments from the cirrhotic rats, suggesting a role for sensory innervation in the decreased vasoconstrictor response to EFS in cirrhosis and, consequently, in splanchnic hyperdynamic circulation. Previously, we have reported that sensory innervation plays a role in hypertension (Balfagón *et al.* 2004), acting as a compensatory mechanism. However, in liver cirrhosis it seems to strengthen hyperdynamic circulation.

This increased participation of sensory innervation might be associated with an increase in the release of CGRP and/or vasodilatation in response to CGRP. Both basal and EFS-induced CGRP releases were higher in mesenteric segments from cirrhotic than control rats, and this agrees with the increase in plasma circulating CGRP levels described in cirrhosis (Bendtsen *et al.* 1991; Møller *et al.* 1996; Henriksen *et al.* 1999). Additionally, the vasodilator response to exogenous CGRP was also higher in segments from cirrhotic rats. An interaction between endothelium and vascular smooth muscle cells has been described. In this sense, we have previously reported that several endothelial factors alter the vasomotor response to EFS (Ferrer *et al.* 2000, 2001, 2004). However, exogenously applied CGRP induced a vasodilator response independent of the presence of endothelial cells (Saito *et al.* 1989). Altogether, these results indicate that sensory innervation involvement in cirrhosis is increased through two different mechanisms, both of which contribute to the decreased contractile response to EFS.

Therefore, our next objective was to determine the mechanism leading to the increased vasodilator response to CGRP. Previously, we reported an increased vasodilator response to CGRP associated with an increase in RAMP1 expression in mesenteric artery (Márquez-Rodas *et al.* 2006a), while changes in CL receptor expression without an increase in RAMP1 do not correlate with changes in the functional role of the CGRP receptor (Márquez-Rodas *et al.* 2008). With this in mind, it is possible that cirrhosis increases the CGRP vasodilator response by upregulating the CGRP receptor component RAMP1. Several tissues have been reported to present co-expression of two different RAMP1 forms (30 and 15 kDa; Sexton *et al.* 2001; Cueille *et al.* 2002). In the present study, we only detected the 30 kDa form, which we have already reported in rat mesenteric artery (Márquez-Rodas *et al.* 2006a). Our results show that cirrhosis did not modify the expression of either the 30 kDa RAMP1 form or the CL receptor protein, indicating that the enhanced vasodilator response to CGRP in the cirrhotic segments corresponded to a downstream signal transduction pathway. For that reason, we performed the following protocols only in segments from cirrhotic rats.

Potassium channels have been implicated in the enhanced vasodilator response to CGRP that has been observed in several pathologies (Balfagón *et al.* 2004; Márquez-Rodas *et al.* 2006b). Calcitonin gene-related peptide exerts a vasodilator action through the activation of K_{ATP} or K_{Ca} channels (Wimalawansa, 1996; Brayden, 2002; Vedernikov *et al.* 2002; de Hoon *et al.* 2003). Thus, experiments were performed in the presence of the K_{ATP} channel antagonist glibenclamide, or in the presence of a combination of charybdotoxin plus apamin, which antagonizes Ca^{2+} -activated potassium

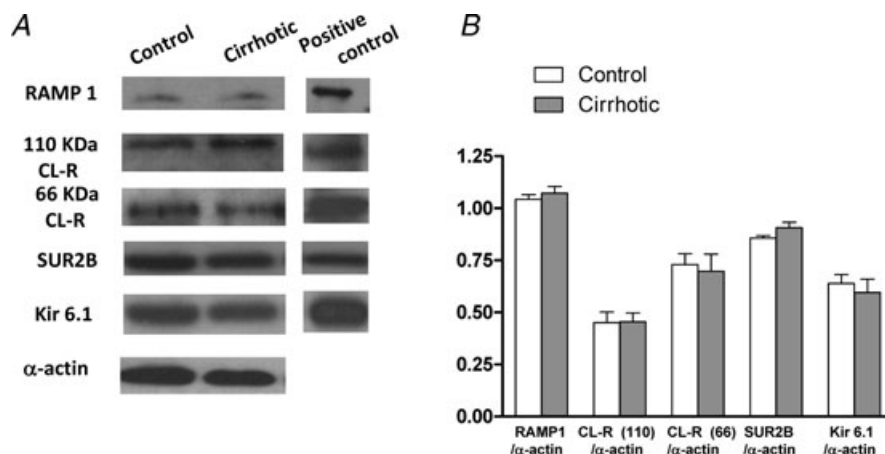


Figure 7. Western blot for RAMP1, CL receptor, SUR2B and Kir 6.1 expression in mesenteric artery segments from control and cirrhotic rats

The figure is representative of preparations from four rats each group. B shows densitometric analysis for protein expression.

channels (K_{Ca} channels). The enhancement of CGRP-induced vasodilatation produced by liver cirrhosis was significantly reduced in the presence of glibenclamide, while charybdotoxin plus apamin did not modify the vasodilator response to CGRP, supporting the participation of K_{ATP} channels and ruling out the participation of K_{Ca} channels in this effect.

Previous studies report an alteration of K_{ATP} channel activity in cirrhosis, an alteration which eventually affects hyperdynamic circulation and splanchnic vasodilatation (Moreau *et al.* 1992, 1994). Consequently, we analysed whether there were any differences in the vasodilator response to the K_{ATP} channel opener pinacidil. The results showed that pinacidil induced a vasodilator response that was greater in cirrhotic than in control animals. This vasodilator response was practically abolished in both experimental groups by incubation with glibenclamide, suggesting that increased K_{ATP} channel sensitivity could mediate the enhanced vasodilator response to CGRP observed in cirrhosis. This increased K_{ATP} channel sensitivity observed in liver cirrhosis may result from either an increase in the gating due to altered subunit expression or from an increase in K_{ATP} current in mesenteric myocytes. Western blot analysis showed no differences in Kir 6.1 and SUR2B expression, while patch-clamp experiments demonstrated an increase in K_{ATP} current in mesenteric myocytes. Similar results have been reported in aorta from Wistar–Kyoto and spontaneously hypertensive rats, where expression of Kir 6.1 and SUR2B did not correlate with changes in the functional role of K_{ATP} channels (Blanco-Rivero *et al.* 2008).

The published results on the role of cAMP and cGMP in the vasodilator response to CGRP are contradictory. While both second messengers have a demonstrable role in this response (Nelson & Quayle, 1995; Vedernikov *et al.* 2002; Brain & Grant, 2004; De Mey *et al.* 2008), we have observed that only cGMP participates in this pathway in rat mesenteric artery (Balfagón *et al.* 2004). Thus, we analysed the effect of RP-cAMP and ODQ, the inhibitors of protein kinase A and guanylate cyclase, respectively, on CGRP-induced vasodilatation in cirrhotic segments. In the present study, the results showed that ODQ practically abolished the vasodilator response to CGRP, while RP-cAMP did not have any effect, confirming that cGMP and not cAMP is involved in this response, as already reported (Balfagón *et al.* 2004). This cGMP participation could result either from an increase in cGMP production or from an increased vasodilator response to cGMP. Our results indicate that cGMP production was not modified in mesenteric arteries from cirrhotic animals. Additionally, the vasodilatation produced by the non-hydrolysable cGMP analogue 8Br-cGMP was higher in segments from cirrhotic rats. These results indicate that the increased response to cGMP is responsible for

increased vasodilatation induced by CGRP in this model of cirrhosis.

In conclusion, these results indicate that the decreased vasoconstrictor response to EFS in cirrhosis is associated with an increased vasodilator response to CGRP and mediated by an enhanced K_{ATP} channel gating. This effect of CGRP may play a role in the splanchnic vasodilatation present in liver cirrhosis.

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