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RESEARCH PAPER

Portal hypertension and liver cirrhosis in rats: effect of the β_3-adrenoceptor agonist SR58611A

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BACKGROUND AND PURPOSE

β_3-Adrenoceptors participate in the regulation of vascular tone in physiological and pathological conditions. We aimed to assess the effect of pharmacological modulation of β_3-adrenoceptors on portal pressure (PP) and systemic haemodynamics and their expression in the liver and mesenteric vessels of cirrhotic rats.

EXPERIMENTAL APPROACH

PP, central venous pressure (CVP) and systemic haemodynamics were invasively assessed in control and CCl_4-treated cirrhotic rats before and during infusion of the selective β_3-adrenoceptor agonist, SR58611A. Tissue samples were also collected from liver, heart, portal vein and mesenteric artery for immunohistochemistry and molecular biology analysis. The effect of SR58611A on isolated portal vein was assessed.

KEY RESULTS

At baseline, cirrhotic rats showed portal hypertension, reduced CVP and hyperdynamic circulation. SR58611A induced a significant, dose-dependent decrease in PP in cirrhotic rats, but not in controls. Although both groups manifested a dose-dependent reduction in mean arterial pressure, this effect was associated with decreased cardiac index (CI) and unchanged indicized peripheral vascular resistance (PVRI) in cirrhotic rats and increased CI and decreased PVRI in control animals. Pretreatment with the selective β_3-adrenoceptor antagonist SR59230 prevented all SR58611A-induced changes in cirrhotic rats. SR58611A concentration-dependently relaxed portal vein in cirrhotic rats to a significantly greater extent than in healthy rats; pretreatment with SR59230A completely prevented SR58611A-induced cirrhotic portal vein relaxation. Finally, β_3-adrenoceptors were identified in the liver, heart and portal vein of cirrhotic and control animals; their expression was increased in cirrhotic rats.

CONCLUSIONS AND IMPLICATIONS

β_3-Adrenoceptors are altered in portal hypertension of experimental cirrhosis and may represent a novel therapeutic target.

Abbreviations

CCl_4, carbon tetrachloride; CI, cardiac index; CO, cardiac output; CVP, central venous pressure; HVPG, hepatic venous pressure gradient; MAP, mean arterial pressure; PP, portal pressure; PVRI, indicized peripheral vascular resistance; SR59230A, 3-(2-ethylphenoxy)-1-[[(1S)-1,2,3,4-tetrahydronaphth-1-yl]amino]-(2S)-2-propanol oxalate salt

Introduction

Portal hypertension is a main feature of liver cirrhosis: it is responsible for the formation of gastro-oesophageal varices, ultimately leading to gastrointestinal bleeding, and contributes to other severe complications, such as ascites, hepatorenal and hepatopulmonary syndromes, and hepatic encephalopathy (Sanyal *et al.*, 2008). Portal hypertension results from the increase in both intrahepatic vascular resistance and, to a lesser extent, portal blood flow. While the latter results from an increased inflow in the splanchnic area secondary to arterial vasodilatation, two major factors concur to the increased intrahepatic vascular resistance: a structural component, represented by the distortion of the liver vascular architecture caused by fibrosis and regenerative nodule formation, and a dynamic component. This results from an imbalance between local vasoactive molecules favouring vasoconstriction and accounts for approximately 25–30% of the total intrahepatic vascular resistance (Bosch *et al.*, 2008).

In clinical practice, portal pressure (PP) is evaluated by determining the hepatic venous pressure gradient (HVPG), with normal values ranging from 3 to 5 mmHg. When HVPG increases above the critical threshold level of 10 mmHg, gastro-oesophageal varices and clinical decompensation can develop (Garcia-Tsao *et al.*, 2007; Bosch *et al.*, 2008). Non-selective β-adrenoceptor blockers are currently indicated by clinical guidelines as the first choice treatment for portal hypertension, as patients whose post-treatment HVPG decreases below 12 mmHg or at least 20% from baseline level ('responder') present not only a lower risk of variceal bleeding but also a lower probability of developing complications such as ascites, spontaneous bacterial peritonitis and hepatorenal syndrome, and death (Garcia-Tsao *et al.*, 2007). β-Blockers lower portal hypertension by decreasing cardiac output, through β_1-adrenoceptor blockade, and splanchnic blood flow, through β_2-adrenoceptor inhibition (Garcia-Tsao *et al.*, 2007; Bosch *et al.*, 2008). However, apart from poor tolerance or the occurrence of side effects, the use of non-selective β-blockers is hampered by the quite heterogeneous HVPG response. Indeed, the prevalence of patients failing to reach the above targets ('non-responders') ranges between 30% and 60%. The causes of this phenomenon still await clarification, even though some hypotheses have been proposed, including down-regulation of β_2-adrenoceptors (Gerbes *et al.*, 1986) or the lower affinity of β_2-adrenoceptors to circulating catecholamines (García-Pagán *et al.*, 1992; Turnes *et al.*, 2006), possibly related to gene polymorphism (Turnes *et al.*, 2006).

Besides the well-studied effects on the β-adrenoceptor subtypes β_1 and β_2, catecholamines can also act through a third β-adrenoceptor subtype, the β_3-adrenoceptor, which differs from the other receptors in its molecular structure and pharmacological profile (Rozec and Gauthier, 2006; Ursino *et al.*, 2009). Although the expression of the β_3-adrenoceptor has been demonstrated in many organs and tissues, including adipose tissue, heart and blood vessels, gastrointestinal tract and liver, urinary bladder and myometrium, its functional role is controversial and only partly established (Berkowitz *et al.*, 1995). β_3-Adrenoceptor stimulation is characterized by a potent vasodilating activity (Rozec and Gauthier, 2006) and participates in the regulation of the vascular tone.

β_3-Adrenoceptor stimulation may represent an alternative pharmacological tool to reduce PP in non-responders or in patients with an adverse reaction to classical β-blockers.

It has been shown that administration of the selective β_3-adrenoceptor agonist SR56811A in healthy animals induces vasodilatation, which is particularly pronounced in the peripheral arterial bed (Montastruc *et al.*, 1999); moreover, the administration of other selective β_3-adrenoceptor agonists induces also vasodilatation in the rat aorta (Montastruc *et al.*, 1999). Recently, Trebicka *et al.* demonstrated that treatment with a partial β_3-adrenoceptor agonist reduces portal hypertension in a rat model of liver cirrhosis, probably acting on intrahepatic resistance with little effect on systemic haemodynamics (Trebicka *et al.*, 2009).

The aims of this study were: (i) to study the effects of the selective β_3-adrenoceptor agonist SR58611A (Giudice *et al.*, 1989; Bianchetti and Manara, 1990; De Ponti *et al.*, 1995; Montastruc *et al.*, 1999) on PP and systemic haemodynamics in an experimental model of liver cirrhosis in the rat; and (ii) to analyse the expression of β_3-adrenoceptors in the liver, heart and splanchnic vasculature of healthy and cirrhotic rats.

Methods

Animal model

Animal care and handling and all experiments were carried out according to the guidelines set forth by EEC Directive 86/609 on the care and use of experimental animals. The protocol for the induction of cirrhosis was approved by the Institutional Ethics Committee of the University of Bologna (Protocol 36402-X/6 dated 27 July 2006). All studies involving animals are reported in accordance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Animals were housed in a controlled environment (22–24°C), maintained on a standard 12-h light/dark cycle (lights on at 07.00 h) and had free access to food and water throughout the study. Male Wistar rats (Charles River Laboratories, Calco, LC, Italy), weighing 175–200 g, were used throughout the study. The total number of animals used in our study was 51 (21 control rats and 30 rats with cirrhosis). Three rats died during the 6–8 week period of CCl_4-induction of cirrhosis; thus, the number of animals used in the experiments was 48.

Induction of cirrhosis

Cirrhosis was induced by carbon tetrachloride (CCl_4) inhalation according to Jiménez *et al.* (1992). Briefly, CCl_4 was used as hepatotoxin, while phenobarbital (0.3 $g \cdot L^{-1}$ in drinking water) was administered to shorten the time required to induce cirrhosis. After 1 week exposure to phenobarbital, inhalation of CCl_4 was started. The rats were placed in a gas chamber (70 × 25 × 30 cm) and compressed air, bubbling through a flask containing CCl_4, was passed into the gas chamber via a flow meter (1 $L \cdot min^{-1}$). Animals were exposed to the gas atmosphere twice a week (on Mondays and Fridays), starting with 0.5 min of bubbling and 0.5 min in the gas atmosphere. Afterwards, the time was increased to 1 min and then by 1 min until 5 min of air flow and 5 min in gas

atmosphere were reached. The mortality rate associated with this induction method was approximately 5–10%.

Experimental design

PP and systemic haemodynamics were invasively assessed in cirrhotic rats with ascites of recent onset, as assessed by physical examination, and in age- and weight-comparable healthy rats (controls). Three experimental groups were used:

- control rats (n = 6)
- cirrhotic rats receiving the β_3-adrenoceptor agonist SR58611A (kindly donated by Sanofi-Aventis, Milan, Italy) (n = 6)
- cirrhotic rats receiving the β_3-adrenoceptor agonist SR58611A, pretreated with the β_3-adrenoceptor antagonist SR59230A (Sigma-Aldrich, Milan, Italy) (n = 6)

In addition, control and cirrhotic rats receiving the β_3-adrenoceptor agonist SR58611A (n = 4) underwent ultrasonographic evaluation in order to evaluate *in vivo* portal vein vasodilatation.

In a separate set of experiments (n = 5), in order to localize and assess β_3-adrenoceptor expression, control and cirrhotic rats were killed to collect tissue samples of liver, mesenteric artery and portal vein. Briefly, tissue samples were removed, washed with PBS and fixed in cold neutral 4% formalin overnight at 4°C. Then, they were placed in 25% sucrose in PBS at 4°C for cryoprotection and embedded in the optimal cutting temperature tissue freezing medium before being processed for immunohistochemistry.

Liver and heart specimens were also snap-frozen in liquid nitrogen and stored at –80°C until subsequent assays.

In a third set of experiments, we assessed the vasorelaxant effect of SR58611A on portal vein specimens, obtained from the control and cirrhotic rats (n = 6 per group).

Haemodynamic studies

The rats from each experimental group were anaesthetized with Zoletil® (Virbac, Milan, Italy), containing zolazepam and tiletamine (ratio 1:1) 10 mg·kg^{-1}, i.m. The depth of anaesthesia was assessed by the pedal withdrawal reflex, recognized as the most reliable for intraoperative care. Respiratory function was monitored and body temperature was maintained (heat lamp) in order to avoid hypothermia.

A PE-50 polyvinyl catheter was placed in the right femoral artery and connected to a transducer (Hewlett Packard, Avondale, PA, USA), which was calibrated before each study, to assess arterial pressure. Another catheter was advanced via the right jugular vein to the right atrium and connected to a spring-loaded syringe (Hamilton Syringe, model CR-700-200; Hamilton Company, Reno, NE, USA) to ensure constant injection rates. A thermocouple (Columbus Instruments, Columbus, OH, USA) was also positioned into the aortic arch, through a left carotid approach, and cardiac output (CO) was measured by thermodilution technique following a bolus of 200 µL of Ringer solution (20–23°C) into the right atrium. CO was calculated by a microcomputer system (Cardiomax III; Columbus Instruments) that simultaneously recorded systolic and diastolic arterial pressures and calculated mean arterial pressure (MAP). Peripheral vascular resistance (PVR) was obtained using the formula PVR = MAP/CO. When not used to assess CO, the catheter placed in the right atrium was connected to a transducer and the central venous pressure (CVP) was measured by using a Power Lab System (PowerLab, AD Instruments Inc., Colorado Springs, CO, USA). Furthermore, a PE-50 polyvinyl catheter was placed in the caecal vein and connected to a transducer to assess PP by using the Power Lab System. The gradient between PP and CVP was calculated in real time by using the Power Lab software. Each value represents the average of two measurements. Finally, the right femoral vein was cannulated to allow the continuous infusion of the drug solution or vehicle by using a syringe pump (Perfusor secura FT; B Braun, Melsungen, Germany).

Haemodynamic parameters were allowed to equilibrate for 30 min and continuously monitored throughout the experiment; afterwards, a continuous infusion of a 0.1% NaCl solution alone (control group) or containing SR58611A was started through the femoral vein and flow speed was changed every 15 min to guarantee a progressive increase in the drug dose (15, 30, 60 and 120 µg·kg^{-1}·min^{-1}) (Manara *et al.*, 1996; Donckier *et al.*, 2001). At the end of each 15 min period, when all parameters were stable, drug infusion was transiently stopped in order to repeat the haemodynamic study.

In some experiments, the β_3-adrenoceptor antagonist SR59230A (De Ponti *et al.*, 1996; Pelat *et al.*, 2003) (1 mg·kg^{-1}) dissolved in 1 mL of 0.9% NaCl solution was given i.v., 30 min before the start of the haemodynamic study. CO and PVR were normalized to rat body weight: cardiac index (CI) and indicized peripheral vascular resistance (PVRI) (Domenicali *et al.*, 2009).

In vitro *studies*

Before tissue collection, rats were killed by inhalant anaesthetic (isoflurane), which is considered an acceptable method for killing rodents according to AVMA Guidelines on Euthanasia (American Veterinary Medical Association, 2007).

Portal vein rings were obtained from the first bifurcation of the splenic vein. The portal vein was occluded with a non-traumatic microvascular clip at the end of exposed segment and quickly removed. All further preparation steps were performed in a sterile hood. Each isolated portal vein segment was first transferred to a Petri-plate containing Krebs-Henseleit physiological solution (KHS; Sigma-Aldrich K3753; 10 mM D-glucose, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 4.7 mM KCl, 118 mM NaCl), with the addition of 2.5 mM $CaCl_2{\cdot}2H_2O$ and 25 mM $NaHCO_3$ continuously aerated with carboxygen mixture gas (95% O_2, 5% CO_2); the remaining connective tissue and fat were carefully removed and fine terminal dissection at both ends was performed under a stereomicroscope. Intact portal vein rings (5–7 mm) were used in a 5 mL organ bath and bathed at 37°C in a gassed (95% O_2, 5% CO_2) KHS.

All drugs used in these experiments were dissolved in distilled water and further dilutions of the drugs were made in KHS. Drug concentrations quoted in the text refer to final organ bath concentrations. Tissues were equilibrated for 45 min before the beginning of the experiments, during which the bathing solution was changed every 15 min. An initial tension of 4 mN was monitored by means of an isometric transducer (Astromed-Grass, Milan, Italy) and adjusted before the experiment was started. Cumulative

concentration–response curves (CRCs) were performed with SR58611A (0.1-0.3-1-3-10-30 μM) in phenylephrine (10 μM)-precontracted tissues by adding the higher concentration as soon as the effect of the previous concentration had reached a plateau (every 3 min). In all samples, phenylephrine (10 μM) was administered before the challenge with the β_3-adrenoceptor agonist, to test viability and establish the maximum contraction of the tissues. After 5 min from the last dose, 10 μM isoprenaline was added in order to assess the maximum β-adrenoceptor-mediated relaxation of the tissue.

After being washed four to five times, each tissue preparation was allowed to rest for 5 min or until its tone returned to the control baseline level, then pretreated with the β_3-adrenoceptor antagonist SR59230A (0.1 μM) for 30 min and CRC experiments were repeated. Finally, after being washed four to five times, isolated portal veins were used as KHS-treated controls.

At the end of the experiment, sodium nitroprusside (10^{-3} M) was used to induce maximal relaxation.

Relaxations in response to SR58611A are expressed as inhibition of phenylephrine-induced contractions (10 μM).

Ultrasonographic study

In a separate set of experiments, four healthy controls and four cirrhotic rats underwent ultrasonographic evaluation. Rats from each experimental group were anaesthetized with Zoletil® (10 mg·kg^{-1}, i.m.) and SR58611A was continuously infused in the right femoral vein according to the same protocol used for the haemodynamic study. Briefly, the abdomen was opened, the portal vein was exposed for up to 3–5 cm (*in situ*) and the probe was leaned directly on the portal vein. Ultrasonography was performed using ESAOTE MYLAB30 (ESAOTE, Genova, Italy), with a linear probe working at 7.5 MHz.

Immunohistochemistry

Sections of tissue (5 μm thick) were cut, serially mounted on glasses and processed for immunohistochemistry. Anti-β_3-adrenoceptor goat polyclonal antibody (1:50) was employed to detect β_3-adrenoceptors. For immunoperoxidase staining, cryoembedded sections were sequentially treated as follows: 1% hydrogen peroxide to block endogenous peroxidase; normal donkey serum in PBS containing 1% Triton® X-100 for 1 h at room temperature in order to block non-specific binding. Sections were incubated with anti-β_3-adrenoceptor primary antibody in a humid chamber at 4°C overnight, rinsed with PBS and sequentially incubated at room temperature with biotinylated anti-goat immunoglobulin (1:200), streptavidin–peroxidase complex and finally with 3,3′-diaminobenzidine tetrahydrocloride. Specimens were mounted with Mowiol® 4-88 reagent and examined with a light microscope (ECLIPSE 90i; Nikon Instruments, Calenzano, Italy) and representative photomicrographs were taken by DS-5 M digital camera (Nikon Instruments). To verify the specificity of immunohistochemical detections, control experiments were performed by omitting the primary antibody and performing the tissue section pre-absorption, incubation of the β_3-adrenoceptor antibody with the β_3-adrenoceptor blocking peptide (2 μg·mL^{-1}).

RT-PCR

To assess the expression of the gene coding for β_3-adrenoceptors, RT-PCR was performed on heart specimens in order to verify whether changes in β_3-adrenoceptor expression could occur in cirrhotic rats as compared with healthy animals. Total RNA was isolated by the Trizol®-chloroform method. Total RNA (5 μg) served as a template for single-stranded cDNA synthesis in a reaction mixture containing random hexamer oligonucleotide primers (0.05 μg·μL^{-1}), 200 U·μL^{-1} of MMLV-reverse transcriptase, 10 mM dNTPs and 5 mM dithiothreitol. cDNA samples were subjected to PCR in the presence of specific rat β_3-adrenoceptor oligonucleotide primers (sense: 5′-CCACCTTGAACTTCGCTACT-3′; antisense: 5′-TTGTGCCTATTGTGAGAGAT-3′; expected size: 372 bp). PCR, consisting of 2 μL of RT products, 2.5 U *Taq* polymerase, 100 μM dNTPs and 0.1 μM oligonucleotide primers, was carried out at the following conditions: denaturation for 30 s at 94°C, annealing for 30 s at 52°C, extension for 45 s at 72°C for 34 cycles and final extension for 7 min at 72°C. The efficiency of RNA extraction, RT and PCR was evaluated by specific sets of oligonucleotide primers for the housekeeping rat β-actin gene (sense: 5′-GCGTGACATTAAAGAGAAG-3′; antisense: 5′-CACTGTGTTGGCATAGAG-3′; expected size: 286 bp). PCR for β-actin was performed at the following conditions: denaturation for 30 s at 94°C, annealing and extension for 30 s at 52°C and 72°C for 30 cycles, and final extension for 7 min at 72°C. Care was taken to verify that the number of PCR cycles for each primer set was in the linear range in order to perform semi-quantitative analysis of PCR products. The amplified cDNA were separated by 2.0% agarose gel electrophoresis in a tris-acetate-EDTA buffer 1X stained with ethidium bromide (0.5 μg·mL^{-1}). cDNA bands were then visualized by UV light and quantified by densitometric analysis with ALPHAVIEW 2.0.1.1 (Alpha Innotech Corporation, San Leandro, CA, USA). The relative expression of β_3-adrenoceptor mRNA was normalized to that of β-actin.

Western blot

A total of 20 mg of liver tissue was homogenized in lysis buffer containing 2 M Tris, pH 7.5; 0.5% Tween 20®; 0.5 M EDTA; and protease inhibitors (1 μg·μL^{-1} aprotinin and pepstatin; 100 mM PMSF). The homogenate was centrifuged at 12 000× *g* for 15 min at 4°C. The supernatant was collected and stored at –80°C until use. Protein concentration was determined using the Bradford method (Quick Start™ Bradford Protein Assay; BIO-RAD, Hercules, CA, USA) and the protein-dye complex absorbance was read using a spectrophotometer at 595 nm. BSA was used as a standard. Protein samples (15 μg) were separated by 10% SDS-PAGE. Proteins were then transferred to nitrocellulose membranes (Hybond ECL; Amersham Biosciences, Glattbrugg, Switzerland) using blotting solution containing 50 mM Tris, 200 mM glycine and 10% v v^{-1} methanol for 2 h and 30 min at 350 mA. Membranes were then blocked in 5% non-fat milk in T-PBS (10% PBS, 0.1% Tween 20) for 1 h at room temperature (25°C) and exposed to goat polyclonal anti-β_3-adrenoceptor (1:100) and rabbit polyclonal anti-β-actin (1:2500) primary antibodies overnight at 4°C. Membranes were washed three times for 10 min with T-PBS and then incubated with HRP-conjugated anti-goat secondary antibody (1:5000) for 1 h and 30 min to

detect β_3-adrenoceptors and with anti-rabbit HRP-conjugated (1:200) secondary antibody to detect β-actin. Blots were developed with SuperSignal West Pico chemiluminescent substrate following the manufacturer's protocol; blot images were digitally acquired by an LAS3000 Imager (Fujifilm Corporation, Stamford, CT, USA) and expression was analysed semi-quantitatively using AlphaView 2.0.1.1 (Alpha Innotech Corporation).

Statistical analysis

Results are expressed as means ± SEM. Statistical analysis was performed using ANOVA for repeated measures and Bonferroni *post hoc* analysis. A *P*-value lower than 0.05 was considered to be significant. Calculations were performed using GraphPad Prism™ software (version 4.0; GraphPad Software Inc., San Diego, CA, USA).

Drugs, chemicals reagents and other materials

SR58611A was kindly donated by Sanofi-Aventis (Milan, Italy). SR59230A, 3,3′-diaminobenzidine tetrahydrocloride, Triton X-100, Tris, Tween 20, EDTA, aprotinin, pepstatin, PMSF, SDS, acrylamide/bis-acrylamide, glycine and methanol were purchased from Sigma-Aldrich.

Anti-β_3-adrenoceptor goat polyclonal antibody (sc-1473) and β_3-adrenoceptor blocking peptide (sc-1473P) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); rabbit polyclonal anti-β-actin antibody was purchased from Chemicon Intetrnational (Hofheim, Germany). Biotinylated anti-goat IgG and HRP-conjugated anti-goat secondary antibody were purchased from Vector Labs (Burlingame, CA, USA). Streptavidin-peroxidase complex and anti-rabbit HRP-conjugated were purchased from Dako Italy (Milan, Italy). Mowiol 4-88 was purchased from Calbiochem (Milan, Italy).

SuperSignal West Pico chemiluminescent substrate was purchased from Pierce Biotechnology (Rockford, IL, USA). The drug and receptor nomenclature used are in accordance with the BJP's Guide to Receptors and Channels (Alexander *et al.*, 2011).

Results

Effect of SR58611A on PP and CVP

PP was significantly higher in cirrhotic than control rats (15.1 ± 1.2 mmHg vs. 7.8 ± 0.5 mmHg; $P < 0.05$). The i.v. administration of SR58611A to cirrhotic rats significantly decreased PP in a dose-dependent manner, reaching approximately 50% of the baseline level with the highest dosage. In contrast, no significant changes occurred in the control rats (Figure 1A). Baseline CVP in cirrhotic rats was lower than that in control rats (–1.4 ± 0.9 mmHg vs. 1.9 ± 0.1 mmHg; $P < 0.05$). CVP was not significantly affected by treatment with SR58611A in either control or cirrhotic rats, even though a modest rise was observed in the latter at the doses of 15 and 30 mg·kg^{-1}·min^{-1} (Figure 1B). As a result, the PP-CVP gradient declined dose-dependently in cirrhotic rats (Figure 1C).

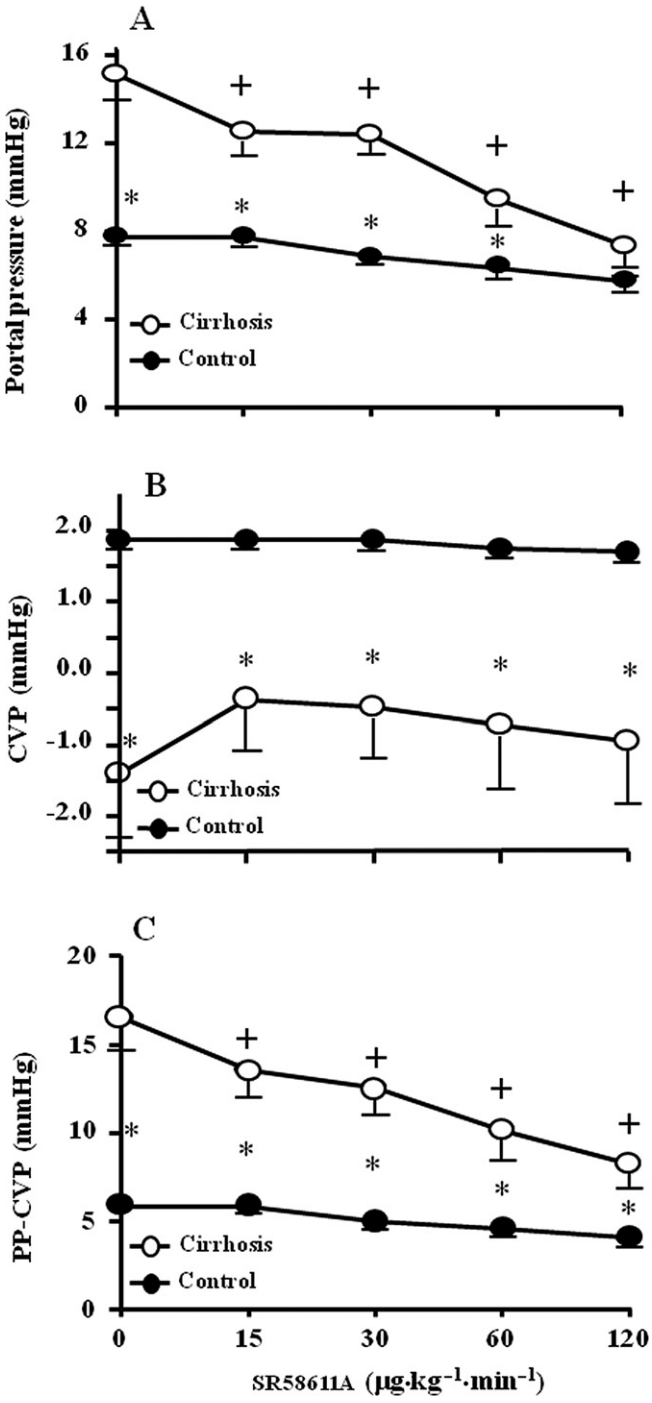

Figure 1

Effect of increasing doses of SR58611A on portal pressure (PP) (A), central venous pressure (CVP) (B) and pressure gradient between PP and CVP (C) in healthy control and cirrhotic rats ($n = 6$, except for the highest dose where $n = 5$). $^{+}P < 0.05$ versus baseline level; $^{*}P < 0.05$ between cirrhosis and healthy controls.

Effect of SR58611A on systemic haemodynamics

Cirrhotic rats presented hyperdynamic circulation, with lower MAP, PVRI and higher CI as compared with control animals (Figure 2).

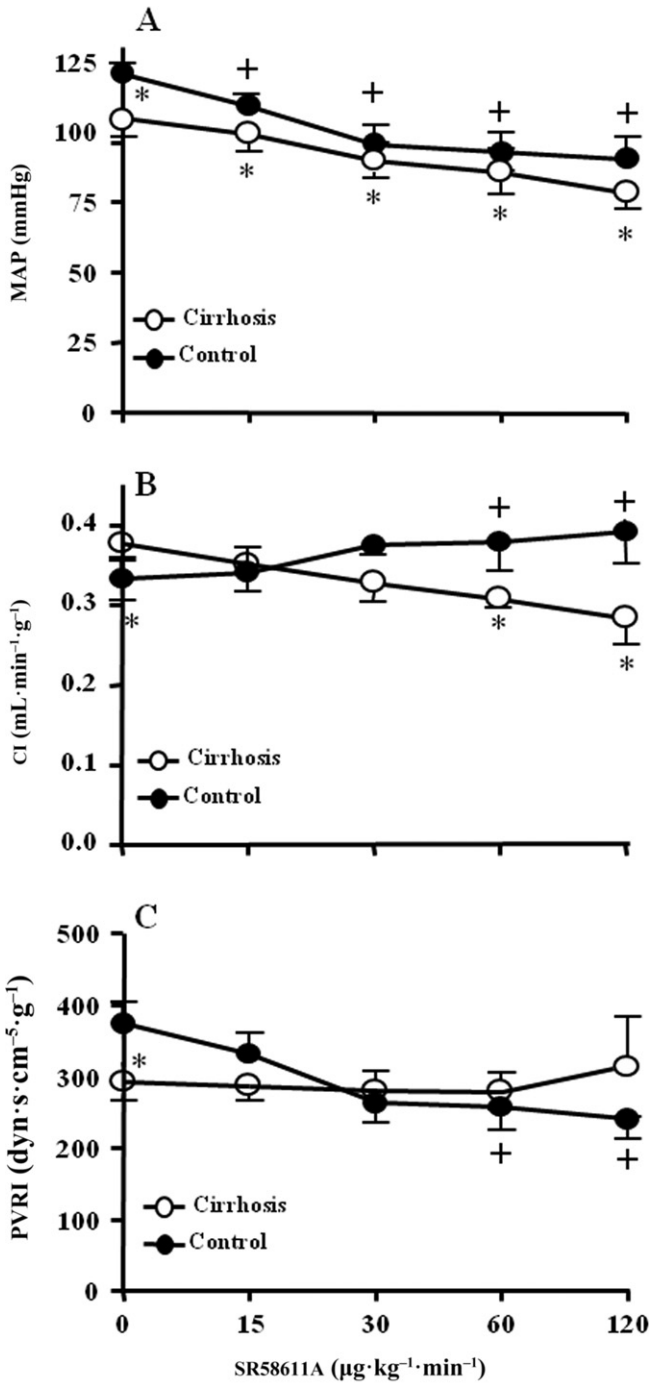

Figure 2

Effect of increasing doses of SR58611A on mean arterial pressure (MAP) (A), cardiac index (CI) (B) and indicized peripheral vascular resistance (PVRI) (C) in healthy control and cirrhotic rats (n = 6, except for the highest dose where n = 5). $^{+}P < 0.05$ versus baseline level; $^{*}P < 0.05$ between cirrhosis and healthy controls.

Treatment with SR58611A significantly and dose-dependently lowered MAP to a similar extent in cirrhotic and control rats (25–30% reduction compared with the baseline level; Figure 2A). However, the effects of SR58611A on CI and PVRI strikingly differed in the two groups: a PVRI decline associated with a rise in CI, which reached the statistical significance with doses of 60 and 120 mg·kg^{-1}·min^{-1}, was observed in healthy controls, while a dose-dependent decrease in CI without any significant change in PVRI occurred in cirrhotic rats (Figure 2B,C).

Effect of pretreatment with SR59230A in cirrhotic rats

Pretreatment of cirrhotic rats with the selective β_3-adrenoceptor antagonist SR59230A fully prevented the changes in PP and systemic haemodynamics induced by increasing doses of the β_3-adrenoceptor agonist SR58611A (Figure 3A–D). Moreover, the administration of SR59230A alone did not affect haemodynamic parameters in cirrhotic rats (data not shown).

Organ bath studies

All portal vein specimens responded to phenylephrine (10 μM) with a sustained contraction.

In healthy rats, SR58611A concentration-dependently relaxed portal vein, with a maximum response of only 8% (92.22 ± 1.15% of phenylephrine-induced contraction; Figure 4); pretreatment with the selective β_3-adrenoceptor antagonist SR59230A had no effect *per se* and did not affect SR58611A-induced relaxation (93.67 ± 1.94% of phenylephrine-induced contraction; Figure 4).

In cirrhotic rats, SR58611A concentration-dependently relaxed portal vein specimens to a significantly greater extent than those from healthy controls, with a maximum response of 16% (84.44 ± 1.88% of phenylephrine-induced contraction; Figure 4); moreover, pretreatment with the selective β_3-adrenoceptor antagonist SR59230A had no effect *per se* and completely prevented β_3-adrenoceptor-induced cirrhotic portal vein relaxation (95.89 ± 1.27% of phenylephrine-induced contraction; Figure 4).

Ultrasonographic study

As shown in Figure 5, ultrasonography indicated an increase in portal vein diameter induced by the selective β_3-adrenoceptor agonist SR58611A at doses of 60 and 120 mg·kg^{-1}·min^{-1} only in cirrhotic rats (3.50 ± 0.12 mm in baseline vs. 4.50 ± 0.34 mm at the highest dose; $P < 0.001$) (Figure 5A). Portal vein diameter was not significantly affected by treatment with SR58611A in control rats (2.48 ± 0.09 mm in baseline vs. 2.53 ± 0.17 mm at the highest dose; P = NS) (Figure 5A).

β_3-Adrenoceptor tissue expression

Immunohistochemical analysis revealed the presence of a low-grade positive immunostaining for β_3-adrenoceptor in liver tissue from control rats, mainly localized in the endothelium of intrahepatic vessels (Figure 6A). β_3-Adrenoceptor immunoreactivity was clearly higher in livers from cirrhotic rats, especially in the perivascular area (Figure 6B).

In the portal vein, the endothelium and muscle cells showed an increased β_3-adrenoceptor immunostaining in cirrhotic animals as compared with healthy controls. β_3-Adrenoceptors were also expressed on perivascular adipocytes, but without any apparent difference between the groups (Figure 6C,D).

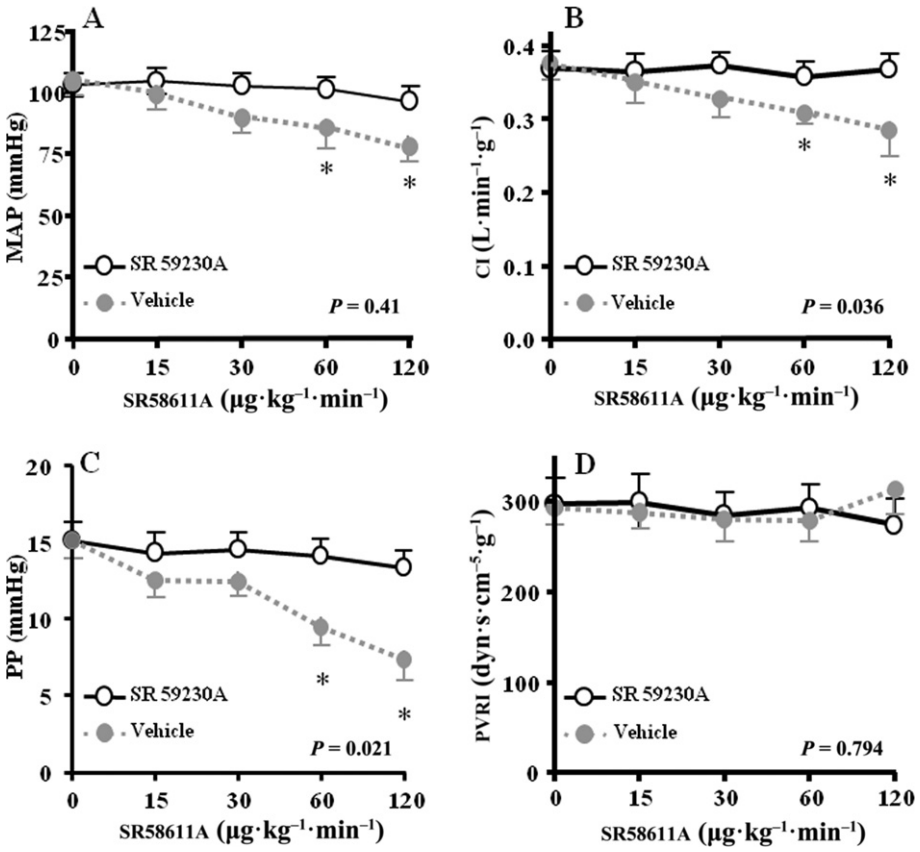

Figure 3

Effect of pretreatment with SR59230A on portal pressure (PP) (A), mean arterial pressure (MAP) (B), cardiac index (CI) (C) and indicized peripheral vascular resistance (PVRI) (D) in cirrhotic rats ($n = 6$, except for the highest dose where $n = 5$) exposed to increasing doses of SR58611A. *$P < 0.05$ between the groups.

Finally, no β_3-adrenoceptor immunoreactivity was seen in the wall of the mesenteric artery isolated from both groups; a low-grade positivity was only present in perivascular adipocytes (Figure 6E,F).

In order to exclude any effect of phenobarbital on β_3-adrenoceptor expression, a separate group of healthy rats was treated with phenobarbital in the drinking water for 2 months. We were not able to find any difference in the β_3-adrenoceptor immunoreactivity with respect to control rats receiving water alone (data not shown).

Western blot analysis showed a several fold increase in β_3-adrenoceptor protein expression in liver tissues from cirrhotic rats (Figure 7A,B).

The RT-PCR analysis showed increased levels of the mRNA encoding for the β_3-adrenoceptor also in the heart tissue of cirrhotic rats as compared with controls (Figure 8).

Discussion and conclusions

The clearest and incontrovertible finding of the present study was that β_3-adrenoceptor stimulation by the selective agonist SR58611A lowered PP in a dose-dependent manner in cirrhotic rats, with a partial effect on portal vein tone. Moreover, CVP was not significantly affected, which implies that the PP-CVP gradient also decreased, which is of utmost importance. In fact, the increase in this gradient, rather that PP *per se*, is associated with portal hypertension-related complications.

Recently, Trebicka *et al.* (2009) demonstrated that treatment with CGP12177A, a partial β_3-adrenoceptor agonist, reduced PP in two different models of cirrhosis. The present study showed that β_3-adrenoceptor stimulation by the selective β_3-adrenoceptor agonist SR58611A significantly decreased PP in cirrhotic rats in a dose-dependent manner; in fact, the highest dose almost halved the baseline values. Selectivity may well account for this different effectiveness. In fact, while SR58611A is highly selective for the β_3-adrenoceptor (Giudice *et al.*, 1989; Bianchetti and Manara, 1990; Manara *et al.*, 1996), CGP12177A is a partial β_3-adrenoceptor agonist with potent β_1- and β_2-adrenoceptor antagonist properties (Vrydag and Michel, 2007). Thus, a lower and less specific effect could be anticipated; indeed, the reduction of PP observed with CGP12177A may be only, in part, attributed to β_3-adrenoceptor stimulation. In our study, the effect of SR58611A was abolished by pretreatment with the selective antagonist SR59230A.

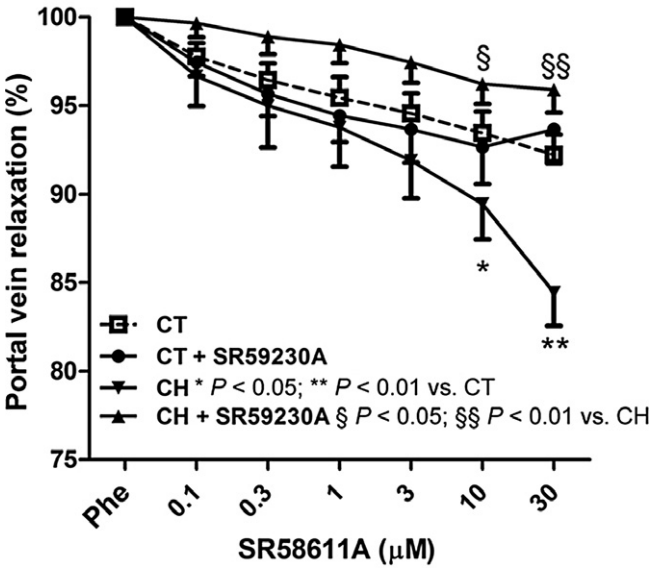

Figure 4

Concentration-dependent responses to SR58611A administered cumulatively (CRC) in healthy control and cirrhotic rats (n = 6 per group), with or without pretreatment with the β_3-adrenoceptor antagonist SR59230A in isolated rings of rat portal vein; *P < 0.05 and **P < 0.01 between cirrhosis + SR58611A and healthy controls + SR58611A; $^{\S}P$ < 0.05 and $^{\S\S}P$ < 0.01 between cirrhosis + SR58611A and cirrhosis + SR58611A pretreated with SR59230A.

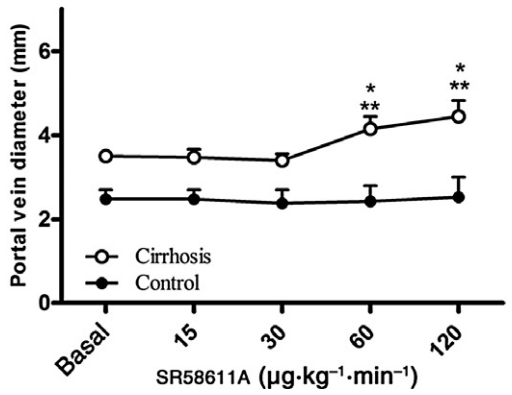

Figure 5

Effect of increasing doses of SR58611A on portal vein vasodilatation in control and cirrhotic rats (n = 4). *P < 0.01 versus baseline level; **P < 0.001 between cirrhosis and healthy controls.

Another interesting finding of this study was that SR58611A administration did not affect PP and portal vein diameter in control rats, as shown by organ bath and ultrasonography experiments. This indicates that irrespective of the mechanism(s) involved, the β_3-adrenoceptor-mediated pathway does not have a prominent role in the regulation of PP under physiological conditions. Contrariwise, the marked effect we observed in cirrhotic rats with severe portal hypertension suggests that this pathway is more sensitive to specific stimulation during pathological conditions such as cirrhosis. This would imply an enhanced receptor expression, and/or post-receptor pathway facilitation, and/or impaired contra-regulation(s).

A reduction in PP in cirrhotic animals or humans can be achieved by either a drop in intrahepatic resistance, or a decrease in splanchnic blood inflow, or both. It has been convincingly demonstrated that β_3-adrenoceptor stimulation leads the vessels expressing this receptor to dilate (Gauthier *et al.*, 2000; Rozec and Gauthier, 2006). We found that β_3-adrenoceptors are highly expressed in the cirrhotic liver, mainly in the perivascular area, supporting the results obtained by Trebicka *et al.* (2009); they showed, in an *in situ* liver perfusion model, that another β_3-adrenoceptor agonist, CPG12177, lowers intrahepatic resistance and PP in rats with cirrhosis induced by bile duct ligation or CCl_4 inhalation. Furthermore, blockade of β_3-adrenoceptors with the selective antagonist SR59230A led to intrahepatic vasoconstriction (Trebicka *et al.*, 2009). These data indicate that the dynamic component of intrahepatic vascular resistance is a major target of β_3-adrenoceptor stimulation and the means through which a reduction in portal hypertension can be obtained.

We also found, for the first time, increased β_3-adrenoceptor expression in the portal vein and a direct SR58611A-induced vasodilatation in the portal vein of cirrhotic rats. Interestingly, the β_3-adrenoceptor also acts as vasorelaxant in the veins (Gauthier *et al.*, 2000; Rozec and Gauthier, 2006). Namely, the activation of β_3-adrenoceptors in rat portal vein myocytes by the agonist CGP12177A stimulates L-type Ca^{2+} channels and promotes vasodilatation, probably modulating smooth muscle tension in vessels (Viard *et al.*, 2000). Thus, this direct vasodilating action of SR58611A on the portal vein may contribute to the reduction in portal hypertension we observed in the present experiments in cirrhotic rats, as has been demonstrated using organ bath and ultrasonography.

As we alluded to above, a reduction in PP can also be achieved by decreasing splanchnic blood inflow, and this has to be taken into account in interpreting our results, as SR58611A is known to be a potent arterial vasodilator (Trochu *et al.*, 1999; Donckier *et al.*, 2001). Consistent with these results, we found that β_3-adrenoceptor stimulation by SR58611A in normal rats actually lowered arterial pressure, as expected. Such a result was mainly due to a reduction in peripheral vascular resistance. Indeed, the CI underwent a significant increase probably aimed at compensating for arterial vasodilatation, but, despite that, arterial pressure declined. β_3-Adrenoceptor stimulation in cirrhotic rats induced substantially different results. In fact, although the final effect on arterial pressure was the same, that is, a significant dose-dependent reduction, the haemodynamic events underlying this change were almost opposite. Namely, the reduction in arterial pressure was associated with a decrease in CI, while peripheral vascular resistance remained steady. It would appear that the cardiac effect was the primary event, evoking a (partial) compensatory response from resistance vessels. β_3-Adrenoceptor stimulation does not evoke cardiac responses under physiological conditions (Ursino *et al.*, 2009). However, the up-regulation of β_3-adrenoceptor-mediated adrenergic system has been proposed as a possible cause of myocardial depression in septic patients (Moniotte *et al.*, 2007). Indeed, increased levels of circulating LPS have

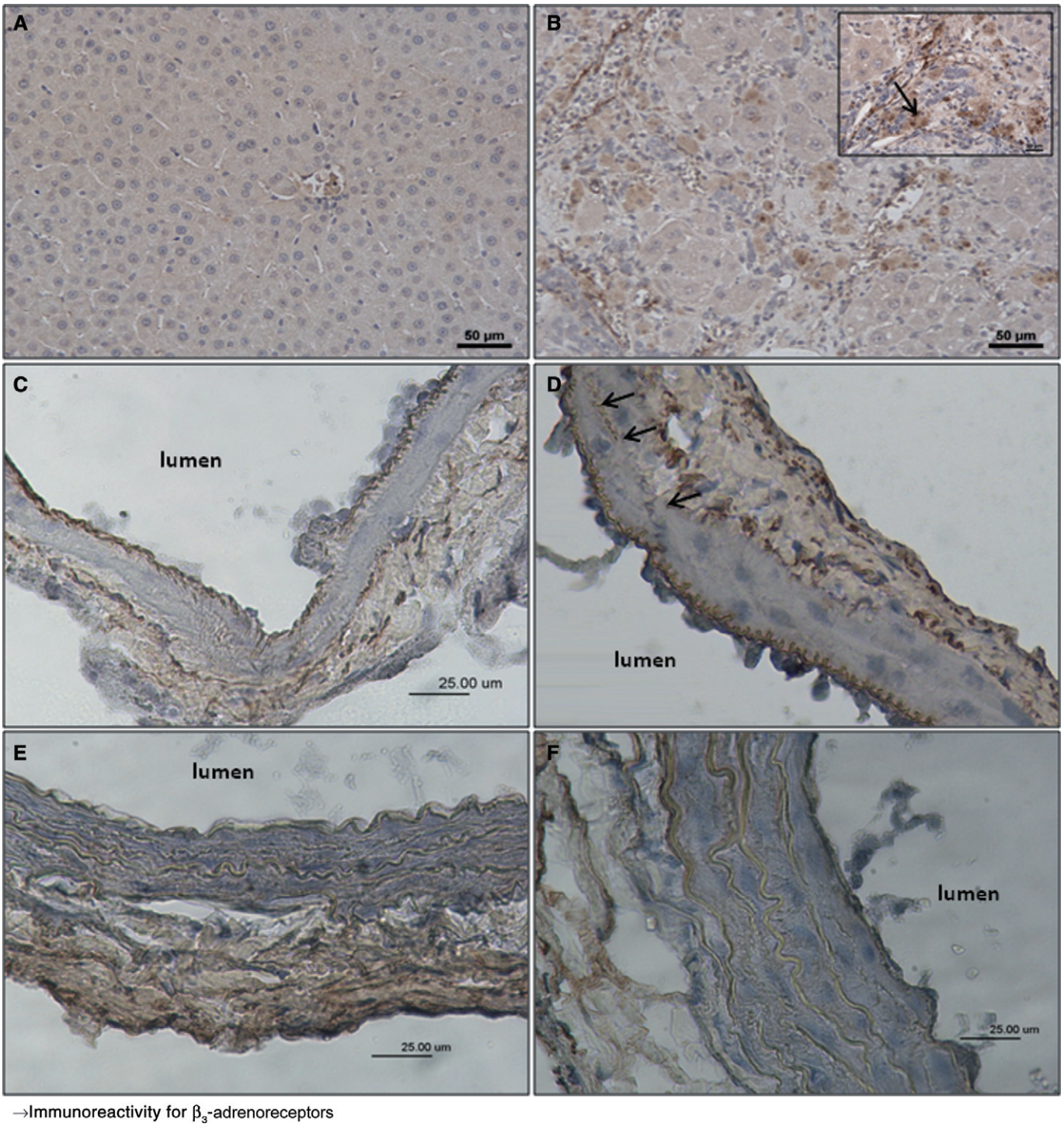

Figure 6

Representative pictures of tissue sections of liver (A, B), portal vein (C, D) and mesenteric artery (E, F) from a healthy control rat (A, C and E) and from a cirrhotic rat (B, D and F). Scale bar: 50 (A–B) and 25 µm.

been reported in both humans (Chan *et al.*, 1997) and experimental animals with cirrhosis (Albillos *et al.*, 2003). In our study, we also observed a significant increase in β_3-adrenoceptor mRNA expression in the heart of cirrhotic rats as compared with healthy rats; it sounds plausible that the up-regulation of cardiac β_3-adrenoceptor gene expression observed in our experiments could be ascribed to chronic increased circulating LPS induced by bacterial translocation. In this context, the administration of a β_3-adrenoceptor agonist like SR58611A would clearly result, especially at

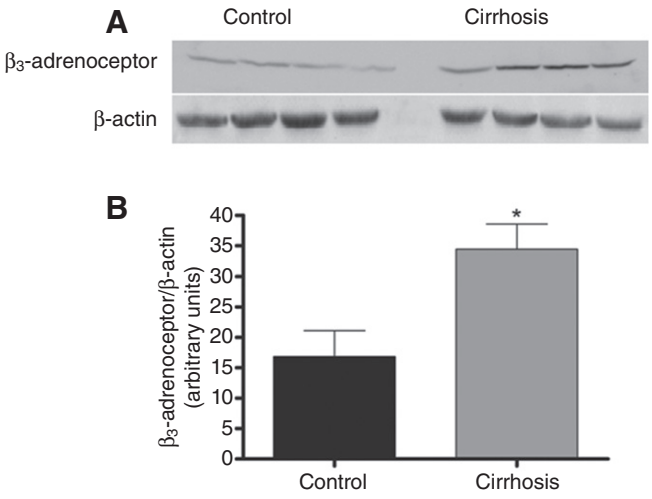

Figure 7

Western blot analysis of β_3-adrenoceptor and β-actin protein in liver tissue from healthy control and cirrhotic rats. (A) Representative SDS-polyacrylamide gel, showing β_3-adrenoceptor (upper line) and β-actin (lower line). (B) Densitometric analysis of β_3-adrenoceptor protein bands normalized to β-actin. Data are expressed as mean ± SEM. $n = 5$. *$P < 0.05$ versus healthy controls.

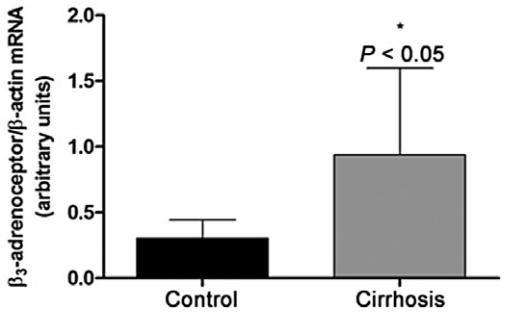

Figure 8

RT-PCR analysis of β_3-adrenoceptor and β-actin mRNA expression in heart tissue from healthy and cirrhotic animals. Column graph refers to the densitometric analysis of β_3-adrenoceptor cDNA bands normalized to the expression of β-actin. Data are expressed as mean ± SEM; $n = 5$, *$P < 0.05$ versus healthy animals.

higher doses, in cardiac inhibition and it must be acknowledged that systemic hypotension and/or cardiac inhibition are potential side effects of β_3-adrenoceptor agonists in cirrhosis, unless liver selective compounds are developed.

Irrespective of the mechanisms underlying the haemodynamic effects of SR58611A, the final result was a reduction in arterial pressure associated with steadiness of peripheral vascular resistance. Thus, a major impact on arterial splanchnic inflow appears unlikely; in any case, a reduction, rather than an increase (as it should be expected to follow the administration of a vasodilator), could have occurred. Moreover, we did not detect any positive immunostaining in the wall of mesenteric arteries isolated from either cirrhotic or normal rats, suggesting the absence of the β_3-adrenoceptor at this level. In line with this, Kozłowska *et al.* showed that β-adrenoceptor agonist-induced vasorelaxation in the rat isolated mesenteric artery was not related to β_3-adrenoceptor stimulation (Kozłowska *et al.*, 2003).

Finally, it should be kept in mind that, at least theoretically, the different effects of SR58611A in health and cirrhosis might be ascribed also to cirrhosis-induced pharmacokinetic changes affecting free fraction of drug, volume of distribution, liver metabolism and urinary elimination. This study was not designed to address these questions.

In conclusion, the present study demonstrates that β_3-adrenoceptor expression is substantially increased in the liver, heart and portal vein of cirrhotic rats. It also provides evidence, for the first time, that the selective agonist SR58611A produces a dose-dependent reduction in PP, which is mainly related to a direct action on portal vein tone; systemic haemodynamic changes, if any, probably play a secondary role. These findings suggest that these receptors may be implicated in the pathogenesis of portal hypertension and may represent a novel pharmacological target in liver cirrhosis.

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Conflicts of interest

None.

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