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Simultaneous inhibition of TxA₂ and PGI₂ synthesis increases NO release in mesenteric resistance arteries from cirrhotic rats

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Abstract

Our study examines, in mesenteric resistance arteries, possible vasodilation alterations, and the role of NO and COX-derivates in cirrhosis. Vasodilator response to acetylcholine was analysed in segments from control and cirrhotic rats. The effects of the unspecific COX inhibitor indomethacin, of the specific COX-1 inhibitor SC-560 and of the specific COX-2 inhibitor NS-398 were analysed in segments from both groups of rats. NO release was measured and eNOS, phosphorylated eNOS (P-eNOS), iNOS, COX-1 and COX-2 expressions were also analysed. The effects of TP receptor antagonist SQ 29548, TxA₂ synthesis inhibitor furegrelate, of PGI₂ synthesis inhibitor TCP or of TCP-plus-furegrelate were only determined in segments from cirrhotic rats. Vasodilator response to acetylcholine was higher in segments from cirrhotic rats. Indomethacin, SC-560 and NS-398 did not modify vasodilator response in control rats. However, indomethacin, NS-398 and TCP-plus-furegrelate increased, SC-560 did not modify, and SQ 29548, furegrelate or TCP decreased the vasodilator response to acetylcholine in cirrhotic rats. NO release was higher in cirrhotic rats. Furegrelate decreased, while TCP-plus-furegrelate increased the NO release in segments from cirrhotic rats. eNOS and COX-1 expression were not modified, while P-eNOS, iNOS and COX-2 expression were higher in cirrhotic rats. The increased iNOS expression and eNOS activity mediate increases in endothelial NO release. The COX-2 derivates TxA₂ and PGI₂ act simultaneously, producing a compensatory effect that reduces NO release and may limit hyperdynamic circulation.

Introduction

Cirrhosis is associated with a hyperdynamic circulatory syndrome characterised by high cardiac output and reduced systemic vascular resistance and arterial pressure. Vascular resistance in circulatory districts other than the splanchnic system can be normal or increased, depending on the degree of portal hypertension, while splanchnic arterial vasodilation produces the hyperdynamic circulatory syndrome. The effects of endothelial factors, mainly nitric oxide (NO) and cyclooxygenase (COX)-derivatives, such as prostacyclin (PGI₂) [1] are reported to be the mechanism(s) leading to the splanchnic vasodilation.

Various endothelial factors are involved in the regulation of vascular resistance in normal and altered haemodynamic conditions and we believe that these factors probably also influence resistance arteries in cirrhosis. NO is synthesised by a family of enzymes, which is divided into two classes: constitutive (cNOS) and inducible (iNOS) [2, 3]. Many investigations have demonstrated that enhanced NO release contributes to the hyperdynamic process occurring in cirrhosis [4, 5], but exactly which NOS isoform(s) and to what extent is still controversial.

There are few studies analysing the role of COX derivatives in cirrhosis. Some of these studies have found PGI₂ release to be altered, and this alteration seems to affect the hyperdynamic circulation observed in cirrhosis [5]. Although enhanced production of vasoconstrictive COX derivatives has been reported to contribute to the increased vascular tone of cirrhotic livers [6], hardly any report has considered their possible role in hyperdynamic splanchnic circulation. NOS and COX are involved in vascular tone regulation and changes in one are accompanied by changes in the other [7].

Thus, our study determines, in mesenteric resistance arteries, possible alterations in vasodilation and the role of NO and COX-derivatives in cirrhosis.

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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 CEE and R.D. 233/88 of the *Ministerio de Agricultura, Pesca y Alimentación* of Spain.

Cirrhosis was induced when rats were 4 months old by intragastric administration of carbon tetrachloride (CCl₄; 0.2 mL/100 g body weight, twice a week; diluted 1:4 in olive oil) along with phenobarbital in the drinking water (0.35 g/L) during 9 weeks, as previously described [8, 9].

Systolic blood pressure was indirectly measured in awake animals by the tail-cuff method [10] (Letica, Digital Pressure Meter, LE5000, Barcelona, Spain). Rats were sacrificed by CO₂ inhalation; the mesenteric vascular bed was removed and placed in cold (4°C) Krebs-Henseleit solution (KHS; in mmol/L: 115 NaCl, 2.5 CaCl₂, 4.6 KCl, 1.2 KH₂PO₄, 1.2, MgSO₄·7H₂O, 25 NaHCO₃, 11.1 glucose, and 0.03 EDTA).

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 portal-systemic collateral circulation. First, a midline abdominal incision with a large bilateral subcostal extension was performed. Mesenteric venous vasculopathy, a characteristic feature of splanchnic venous congestion, was observed as dilation and tortuosity of the superior mesenteric vein branches [11].

The portosystemic collateral circulation was studied by macroscopic examination of the areas in which the collateral venous circulation had developed (splenorenal, gastroesophageal, colorectal and liver hilum), carefully identifying the development of collateral veins [12].

Lipid and transaminase levels

Blood samples were collected by cardiac puncture before sacrifice. After 15 minutes of centrifugation at 1,500 g, the serum was transferred to polypropylene tubes and then frozen at -80°C. The serum levels of liver transaminases GOT and GPT, cholesterol, triglycerides, HDL and LDL lipoproteins, were measured by spectrophotometric colorimetric techniques, according to the manufacturer instructions (SpinReact, SA, Gerona, Spain).

Vascular reactivity study

For vascular reactivity experiments the third order branch of the mesenteric arcade (Control: 318 ± 6.3 vs. Cirrhotic: 310 ± 9.7 µm internal diameter, P>0.05) was dissected from the mesenteric bed, cleaned of connective tissue and cut into segments of approximately 2 mm in length. Two tungsten wires (40 µm diameter) were introduced through the lumen of the segments and mounted in a small vessel chamber myograph (Danish Myo Technology A/S, Århus, Denmark) to measure isometric tension according to the method described by Mulvany and Halpern [13]. After a 30 min equilibration period in oxygenated (95%O₂/ 5% CO₂) KHS at 37 °C and pH 7.4, segments were stretched to their optimal lumen diameter for active tension development. This was determined based on the internal circumference-wall tension ratio of the segments by setting their internal circumference, L₀, to 90% of what the

vessels would have if they were exposed to a passive tension that was equivalent to the tension produced by a transmural pressure of 100 mmHg [13].

Experimental Protocols

After a 30 min equilibration period, arteries were exposed twice to 120 mmol/L KCl to check their functional integrity. Afterwards, concentration-response curves to acetylcholine (1 nmol/L - 1 μ mol/L) were made in arterial segments previously contracted with noradrenaline at a concentration that produced close to 50% of the contraction induced by KCl (120 mmol/L). This curve was performed in absence and in presence of the NO synthase inhibitor L-NAME (100 μ mol/L).

To determine the role of COX derivates in the vasodilator response to acetylcholine, either the unspecific COX inhibitor indomethacin (10 μ mol/L), the constitutive COX-1 inhibitor SC-560 (50 nmol/), or the inducible COX-2 inhibitor, NS-398 (10 μ mol/L), were added to the organ chamber 30 min before the concentration-response curve to acetylcholine was performed. In another set of experiments, segments were incubated with the TP receptor antagonist SQ 29548 (1 μ mol/L), with the PGI₂ synthesis inhibitor tranylcypromine (TCP, 10 μ mol/L), with the thromboxane A₂ (TxA₂) synthase inhibitor, furegrelate (1 μ mol/L), or with a combination of both TCP and furegrelate, to analyze the possible involvement of these vasoactive factors in relaxation to acetylcholine.

To assess possible differences in NO vasodilator response and TxA₂ vasoconstrictor response, concentration-response curves to either the NO donor dyethylamine NONOate (DEA-NO, 0.1 nmol/L-0.1 mmol/L) or the TxA₂ mimetic U46619 (0.1 nmol/L-3 μ mol/L) were performed in segments from control and cirrhotic animals.

Nitric Oxide release

NO release was measured as previously described [14]. In order to study the effect of acetylcholine on NO release the second, third and fourth branches of mesenteric artery were incubated for 60 minutes in 3 mL of HEPES buffer, containing in mmol/L: 119 NaCl, 20 HEPES, 4.6 KCl, 1 MgSO₄.7H₂O, 0.15 Na₂HPO₄.12H₂O, 0.4 KH₂PO₄, 5 NaHCO₃, 1.2 CaCl₂.2H₂O, 5.2 glucose, at 37°C (stabilisation period). Afterward, arteries were incubated with the fluorescent probe 4,5-diaminofluorescein (2 μ mol/L) for 45 min and medium was collected to measure basal NO release. Once the organ bath was refilled, a dose-response curve to acetylcholine was performed, as described in the *Experimental Protocol*. The medium was collected only at the end of the concentration-response curve to acetylcholine. The fluorescence of the medium was measured at room temperature using a spectrofluorimeter (LS50 Perkin Elmer Instruments, FL WINLAB Software) with excitation wavelength set at 492 nm and emission wavelength at 515 nm. The stimulated NO release was calculated by subtracting the basal NO release from that evoked by acetylcholine. Also, blank measurement samples were collected from medium without resistance mesenteric segments in order to calculate and subtract the background emission. Some assays were performed in the presence of L-NAME (0.1 mmol/L), TCP (10 μ mol/L), furegrelate (1 μ mol/L) or TCP-plus-furegrelate to assess the effect of these drugs on NO release, but only in segments from cirrhotic rats. The amount of NO released was expressed as arbitrary units mg tissue⁻¹.

Detection of superoxide anions

In order to study the effect of liver cirrhosis on superoxide anion release, the second, third and fourth branches of mesenteric artery from either control or cirrhotic

rats were equilibrated for 30 min in HEPES buffer at 37°C, transferred to test tubes that contained 1 mL HEPES buffer (pH 7.4) containing lucigenin (250 µmol/L) and then kept at 37°C. The luminometer was set to report arbitrary units of emitted light; repeated measurements were collected during 5 min at 10 s intervals and averaged. 4,5-Dihydroxy-1,3-benzene-disulphonic acid "Tiron" (10 mmol/L), a cell permeant and non-enzymatic scavenger of the superoxide anion, was added to quench the superoxide anion-dependent chemiluminescence. Also, blank measurement samples without mesenteric segments were collected in the same way to subtract background emission. The amount of superoxide anion released was expressed as chemoluminescence units min⁻¹ mg tissue⁻¹.

Prostanoids and CRP (C-reactive protein) production

To measure the release of CRP, and the stable metabolites of TXA₂ and PGI₂, TXB₂ and 6-keto PGF_{1α} respectively, a rat CRP ELISA kit (BD Biosciences), TXB₂ and 6-keto-PGF_{1α} EIA kits (Cayman Chemical) were used. The second, third and fourth branches of mesenteric resistance arteries from control and cirrhotic rats were preincubated for 45 minutes in 200 µL of KHS at 37°C and continuously gassed with a 95% O₂ - 5% CO₂ mixture (stabilisation period). Afterward, three washout periods of seven minutes in a bath of 200 µL of KHS were run before incubation with acetylcholine (0.1 nmol/L - 0.1 µmol/L). The medium was collected only at the end of concentration-response curve to acetylcholine. Some samples were collected in the presence of 1 µmol/L furegrelate in order to determine possible variations produced by this inhibitor. The different assays were performed following the manufacturer's instructions. Results were expressed as ng CRP mL⁻¹ mg tissue⁻¹, or pg.prostanoid mL⁻¹ mg wet tissue⁻¹.

Western blot

For Western blot analysis, the second, third and fourth branches of mesenteric artery from both groups of rats were homogenised in a boiling buffer composed of 1 mmol/L sodium vanadate, 1% SDS, and 0.01 mol/L pH 7.4 Tris-HCl. Homogenates containing 30 µg protein were electrophoretically separated on a 7.5% SDS-polyacrylamide gel, 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 Laboratories, Hercules, CA, USA) containing 25 mmol/L Tris, 190 mM glycine, 20% methanol, and 0.05% SDS. The membrane was blocked for 1 h at room temperature in a Tris-buffered-saline (TBS) solution (100 mmol/L Tris-HCl, pH 7.4, 0.9% w/v NaCl, 0.1% SDS) with 5% powdered fat-free milk before being incubated overnight at 4°C with mouse monoclonal antibody against iNOS (1:5000 dilution, Transduction Laboratories), mouse monoclonal antibody against eNOS (1:2500 dilution, Transduction Laboratories) rabbit polyclonal antibody against phosphorylated eNOS (P-eNOS, assessed by its phosphorylation at serine 1177, 1:500, Abcam Laboratories), mouse monoclonal antibody against COX-1 (1:500, Cayman Chemical), or rabbit polyclonal antibody against COX-2 (1:500, Cayman Chemical). After washing, the membrane was incubated with the appropriate secondary horseradish peroxidase-conjugated immunoglobulin G antibody (Amersham International Plc). The membrane was thoroughly washed and the immunocomplexes were detected using an enhanced horseradish peroxidase/luminol chemiluminescence system (ECL Plus, Amersham International Plc, Little Chalfont, UK) and subjected to autoradiography (Hyperfilm ECL, Amersham International Plc). Signals on the immunoblot were quantified using a computer program (NIH Image V1.56, National Institute 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 antibody anti α -actin (1:2000 dilution, Sigma).

Drugs and Solutions

The drugs used were L-NA hydrochloride, acetylcholine chloride, diethylamine NONOate diethylammonium salt, U46619, SQ 29548, L-NAME hydrochloride, indomethacin, TCP, NS-398, SC-560, SQ 29548, furegrelate, tiron and lucigenin. Stock solutions (10 mmol/L) of drugs were made in distilled water except for noradrenaline, which was dissolved in a NaCl (0.9%)-ascorbic acid (0.01% w/v) solution; indomethacin, U46619, SQ 29548 and SC-560, which were solubilized in ethanol and administered from a prepared stock in such a way that the maximal ethanol concentration of the medium was less than 0.001% (vol./vol.); and NS-398, which was solubilised in DMSO. All stock solutions were kept at -20°C , and appropriate dilutions were made in KHS on the day of the experiment.

Statistical Analysis

Results are expressed as mean \pm SEM for the number of rats indicated. Statistical analysis compared the curve obtained in the presence of the different substances with the control curve by means of two-way analysis of variance (ANOVA). For the NO, prostanoids and CRP release experiments, the statistical analysis employed Student's t test for unpaired experiments. A P value of less than 0.05 was considered significant.

Results

Animal evolution

Liver cirrhosis did not modify systemic blood pressure, but body weight was lower in cirrhotic animals. All cirrhotic animals showed spleen hypertrophy and hepatomegaly (Table 1). The liver histology of cirrhotic animals showed a finely granulated surface and the histological features of cirrhosis (Data not shown).

In the liver cirrhosis group, rats presented portosystemic collateral circulation (pararectal, paraesophageal, splenorenal and portohepatic collateral vessels).

Both GPT and GOT levels were increased in our experimental model. Additionally, LDL levels increased, HDL and triglycerides decreased, and total cholesterol levels were not modified after the establishment of liver cirrhosis (Table 2).

Vascular reactivity experiments

To provide maximal activation for each preparation segments were contracted with 120 mol/L KCl; responses obtained were similar in vessels from control and cirrhotic rats (Control: 2.81 ± 0.11 vs. Cirrhotic: 2.88 ± 0.15 mN/mm, $P > 0.05$). Acetylcholine caused a cumulative concentration-dependent relaxation in noradrenaline-precontracted rat mesenteric resistance arteries (MRA) that was greater in segments from cirrhotic rats (Figure 1); in vessels from both groups, preincubation with L-NAME significantly reduced this response (Figure 1). In presence of L-NAME, relaxation to acetylcholine was similar in vessels from both control and cirrhotic rats (Figure 1)

Pretreatment with indomethacin, SC-560 or NS-398 did not modify vasodilator response in mesenteric segments from control rats (Figure 2A). However, both indomethacin and NS-398 increased vasodilator response to acetylcholine, while SC-560 did not modify the acetylcholine-induced vasodilation in segments from cirrhotic rats (Figure 2B).

Preincubation with SQ 29548, furegrelate or TCP decreased vasodilator response in segments from cirrhotic rats (Figure 3). Simultaneous preincubation with both TCP and furegrelate together increased concentration-response vasodilation to acetylcholine in segments from cirrhotic rats (Figure 3). In arteries from control rats, relaxation to acetylcholine remained unmodified in the presence of SQ 29548, furegrelate, TCP or TCP-plus-furegrelate (results not shown).

Vasodilator response to DEA-NO and vasoconstrictor response to U46619 were similar in both experimental groups (Data not shown).

Nitric oxide release

Both basal and acetylcholine-induced NO release were higher in segments from cirrhotic rats (Figure 4). Preincubation with TCP did not modify either basal or acetylcholine-stimulated NO release in segments from cirrhotic rats (Figure 4). Furegrelate decreased both basal and acetylcholine-induced NO release in segments from cirrhotic rats (Figure 4). A combination of TCP-plus-furegrelate increased both basal and acetylcholine-induced NO release in segments from cirrhotic rats (Figure 4).

Superoxide anion release

Liver cirrhosis increased superoxide anion release by mesenteric resistance arteries (Control: 10.50 ± 1.85 U min⁻¹ mg tissue⁻¹; Cirrhotic: 29.25 ± 3.12 U min⁻¹ mg tissue⁻¹; $P < 0.05$; $n=5$ in each group).

CRP release

Basal CRP release was increased by liver cirrhosis (Control: 4.083 ± 0.78 ng ml⁻¹ mg tissue⁻¹; Cirrhotic: 23.20 ± 6.65 ng ml⁻¹ mg tissue⁻¹; $P < 0.05$; $n=5$ in each group). Acetylcholine increased CRP release in both groups, but the release was higher in segments from cirrhotic rats (Control: 14.93 ± 3.48 ng ml⁻¹ mg tissue⁻¹; Cirrhotic: 47.67 ± 12.27 ng ml⁻¹ mg tissue⁻¹; $P < 0.05$; $n=5$ in each group).

Prostanoids release

Liver cirrhosis did not modify basal, while decreased acetylcholine-induced TxB₂ release in mesenteric resistance arteries (Figure 5A). Both basal and acetylcholine-induced 6-keto PGF_{1α} levels were decreased by liver cirrhosis (Figure 5B). Preincubation with furegrelate did not modify 6-keto PGF_{1α} levels in mesenteric segments from cirrhotic rats (Results not shown).

Western blot

Liver cirrhosis did not modify eNOS or COX-1 expression (Figure 6), while increased P-eNOS, iNOS and COX-2 expression (Figure 6).

Discussion

CCl₄ treatment was maintained for 9 weeks. All CCl₄-treated rats used in these experiments macroscopically showed macro/micronodular cirrhosis of the liver, hepatomegaly and spleen hypertrophy. No rat showed ascites, which has only been described after 12-20 weeks CCl₄ administration in these animals [15]. Transaminase levels (GOT and GPT) were increased after CCl₄ treatment. Additionally, in our experimental model, HDL and triglycerides levels were decreased, LDL increased but total cholesterol was not modified in serum samples. These data agree with previous studies performed in portal hypertensive rats [16]. All these alterations confirm that our experimental model develops liver cirrhosis.

In this study, we have observed that, as reported in conductance mesenteric arteries [17], cirrhosis increases vasodilation induced by acetylcholine in mesenteric resistance arteries. This effect is associated with an increase in NO release while the vasodilator response to DEA-NO remained unmodified, confirming the major role played by NO release in the development of hyperdynamic circulation. This effect was accompanied by an increase in iNOS expression, as previously described in aorta and superior mesenteric arteries [18]. Additionally, in spite of a non-modification in eNOS expression, we observed that eNOS phosphorylation at serine 1177 was increased in cirrhotic rats, indicating increased eNOS activation produced by liver cirrhosis. These observations do not agree with previous reports of both a decrease and a no modification in superior mesenteric artery eNOS expression [17, 18], and a decrease on eNOS activity in aorta and superior mesenteric artery [18] has also been described. This discrepancy can be due to a difference on the vascular bed analysed.

Vascular alterations could be a consequence of oxidative stress [19, 20], so we analysed the release of superoxide anions in our experimental conditions. The results obtained showed, as has been observed in cirrhotic livers [21], an increase on superoxide anion levels in mesenteric segments from cirrhotic rats.

One of the most commonly used inflammatory markers is CRP, which can predict a risk of coronary events [22, 23]. This marker has been reported to rise in short-time PH with and without cirrhosis [24]. The increased CRP levels observed in our experimental conditions confirm an increase in inflammatory processes due to liver cirrhosis.

Since a number of studies have already focused on the role of COX-derived PGI₂ and TxA₂ in the inflammatory process observed in cirrhosis [5, 25], the next objective was to analyse the possible participation of COX-derivates in the vasodilator response induced by acetylcholine in mesenteric resistance arteries from cirrhotic rats. Indomethacin, NS-398 or SC-560 do not have any effect on the vasodilator response in control segments, indicating the non-participation of COX-derivates in this experimental condition, as previously reported [26], while vasodilation increases in cirrhotic segments preincubated with indomethacin or NS-398, but not with SC-560. These results therefore indicate that COX-2-derived arachidonic acid metabolites modulate the endothelium-dependent relaxation in mesenteric resistance arteries from cirrhotic rats, but not from healthy ones. Supporting these results is the fact that COX-2 is overexpressed in segments from cirrhotic rats while COX-1 expression is not modified. Taken all together these results

support the existence of an inflammatory process that would underlie the development of liver cirrhosis [22].

Our next objective was to analyze which COX-2 derivate was implicated in this effect. In our experimental model we have seen that PGI₂ levels increased after the establishment of liver cirrhosis, as has been reported regarding circulating PGI₂ levels in cirrhotic patients and portal hypertensive rats [27, 28]. Numerous studies have demonstrated that PGI₂ promotes vasodilation in various vascular beds by stimulating IP receptors and thereby increasing the intracellular cyclic-AMP concentration. In mesenteric arteries from cirrhotic rats the relaxation to acetylcholine was diminished by incubation with the PGI₂ synthesis inhibitor TCP, indicating that PGI₂ has a functional role on the vasodilator response to acetylcholine and would consequently participate in the increased vasodilation associated to cirrhosis. NO release was not modified by TCP treatment, thus ruling out possible NO participation in this response. This result contrasts with early reports in a similar experimental model that indicated a minor role for PGI₂ in the development of hyperdynamic circulation [29].

Changes in TxA₂ release have also been suspected in cirrhosis. In the present work we have observed that liver cirrhosis decreases TxA₂ levels. Additionally, the TxA₂ analogue U46619 induces vasoconstriction through TP receptor activation. Incubation with TP receptor antagonist SQ 29548 increases the vasodilator response to acetylcholine in the cirrhotic arteries, suggesting that TxA₂ could participate as a vasoconstrictor in the response to this endothelium-dependent vasodilator agent. However, inhibition of TxA₂ synthesis by furegrelate induces a decrease in the vasodilator response to acetylcholine, suggesting that inhibition of TxA₂ synthesis induces some other mechanism that would affect the vasomotor response to acetylcholine. Interactions between COX derivatives are known to be complex; the synthesis of one prostanoid is usually accompanied by changes in the synthesis of the others. This imbalance can be shifted towards hypo- or hyper-compensatory responses [30]. Therefore we analysed the release of the PGI₂ metabolite 6-keto PGF_{1α} after preincubation with furegrelate in segments from cirrhotic rats. Our results show that 6-keto PGF_{1α} release was not modified by preincubation with furegrelate, ruling out the possibility that furegrelate had induced a new equilibrium in prostanoid synthesis in segments from cirrhotic animals. Increasing evidences indicate that there is considerable "cross talk" between the NO and COX-derivate biosynthetic pathways, resulting in an active modulation in several situations [7, 31]. These data lead us to hypothesize a possible modulation of NO synthesis by TxA₂ in arteries from cirrhotic rats. Our results indicate that NO release was diminished in the presence of the TxA₂ synthesis inhibitor furegrelate, thus explaining the decrease of vasodilator response to acetylcholine after TxA₂ synthesis inhibition.

These last results seem to show that both COX-2 derivatives, PGI₂ directly, and TxA₂ indirectly by increasing NO formation, play a role in maintaining the hyperdynamic circulation observed in resistance mesenteric arteries from cirrhotic rats; the results also suggest that inhibition of both TxA₂ and PGI₂ would have an additive effect. Thus, our next objective was to analyse the effect of the simultaneous inhibition of both TxA₂ and PGI₂ synthesis on the response to acetylcholine. Surprisingly, we observed that the relaxation induced by acetylcholine was significantly increased in the presence of TCP-plus-furegrelate.

In line with this possible COX-NO interaction we hypothesised that NO release would increase if TxA_2 and PGI_2 synthesis were simultaneously inhibited. Our hypothesis was confirmed: preincubation with furegrelate combined with TCP increased NO release, and this would explain the increase in the vasodilator response to acetylcholine.

Additionally, the fact that simultaneous TxA_2 and PGI_2 synthesis inhibition produces exactly the same effect on the vasodilator response to acetylcholine as COX-2 inhibition excludes the participation of the other COX-2 derivatives in this effect. To our knowledge, this is the first time that the joint action of two COX-2 derivatives, PGI_2 and TxA_2 , has been described to induce a different effect on NO release than either of them alone. Additionally, these results indicate that activation of both NOS and of COX-2 plays a relevant role in the modulation of vasodilator response to acetylcholine in cirrhosis, and that they lead to opposite effects: while NO seems to participate in hyperdynamic circulation development, the COX-2 derivatives TxA_2 and PGI_2 act together, seeming to produce a compensatory mechanism inhibiting NO.

The use of COX inhibitors like indomethacin has been suggested to avoid liver fibrosis as well as to attenuate the increased portal hypertension produced in liver cirrhosis [32, 33]. However, our results show that, as well as these beneficial effects, the use of COX inhibitors could worsen the hyperdynamic circulation associated with liver cirrhosis.

In conclusion, the present study shows that in mesenteric resistance arteries from cirrhotic rats increased iNOS expression and eNOS activity mediate increases in endothelial NO release. The COX-2 derivatives TxA_2 and PGI_2 act simultaneously, producing a compensatory effect that reduces NO release and could limit the hyperdynamic circulation.

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Table 1

Effect of liver cirrhosis on body weight, systemic blood pressure, spleen weight and liver weight.

	<i>Body weight (g)</i>	<i>Blood pressure (mm Hg)</i>	<i>Spleen weight/ Body weight (%)</i>	<i>Liver weight/ Body weight (%)</i>
<i>Control</i>	525.5 ± 6.4	149.6 ± 4.1	0.1 ± 0.008	3 ± 0.1
<i>Cirrhotic</i>	487.9 ± 6.9 *	144.4 ± 3.4	0.2 ± 0.009 *	4.1 ± 0.1 *

Results are expressed as mean ± S.E.M. * P< 0.05 vs. control. N= 10 animals each group.

Table 2

Effect of liver cirrhosis on GPT, GOT, LDL, HDL, triglycerides and cholesterol levels

	<i>GPT (U/L)</i>	<i>GOT (U/L)</i>	<i>LDL (mg/dL)</i>	<i>HDL (mg/dL)</i>	<i>Triglycerides (mg/dL)</i>	<i>Cholesterol (mg/dL)</i>
<i>Control</i>	53.20 ± 4.9	92.50 ± 6.6	5.25 ± 0.4	31.73± 4.2	58.40 ± 5.2	92.00 ± 7.1
<i>Cirrhotic</i>	100.30 ± 11.8 *	135.20 ± 14.6*	10.80 ± 0.8*	20.94 ± 1.2 *	34.20 ± 4.6*	86.80 ± 9.9

Results are expressed as mean ± S.E.M. * P< 0.05 vs. control. N= 10 animals each group.

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Figure legends

Figure 1. Vasodilator response to acetylcholine in mesenteric resistance arteries from control and cirrhotic rats in the absence and presence of 100 $\mu\text{mol/L}$ L-NAME. Results are expressed as mean \pm S.E.M. Number of animals used is indicated in parentheses.

Figure 2. Effect of preincubation with 10 $\mu\text{mol/L}$ indomethacin, 10 $\mu\text{mol/L}$ NS-398 or 50 nmol/L SC-560 on the vasodilator response to acetylcholine in mesenteric resistance arteries from control (A) and cirrhotic (B) rats. Results are expressed as mean \pm S.E.M. Number of animals used is indicated in parentheses.

Figure 3. Effect of preincubation with 1 $\mu\text{mol/L}$ furegrelate, 10 $\mu\text{mol/L}$ TCP, TCP-plus-furegrelate, or 1 $\mu\text{mol/L}$ SQ 29548 on the vasodilator response to acetylcholine in mesenteric resistance arteries from cirrhotic rats. Results are expressed as mean \pm S.E.M. Number of animals used is indicated in parentheses.

Figure 4. Basal and acetylcholine-induced NO release in segments from control and cirrhotic rats. Effect of TCP (10 $\mu\text{mol/L}$), furegrelate (1 $\mu\text{mol/L}$), or TCP-plus-furegrelate on the basal- and acetylcholine-induced NO release in mesenteric artery segments from cirrhotic animals. Results (Means \pm S.E.M.) were expressed as arbitrary units (A.U.) mg tissue^{-1} . $n = 4-7$ each experimental condition. * $P < 0.05$ vs. Basal; # $P < 0.05$ vs. control rats; # $P < 0.05$; + $P < 0.05$ vs. cirrhotic rats.

Figure 5. Effect of liver cirrhosis on basal and acetylcholine-induced TxB_2 (A) and 6-keto $\text{PGF}_{1\alpha}$ (B) release. Results (Means \pm S.E.M.) were expressed as $\text{pg mL}^{-1} \text{mg tissue}^{-1}$. $n=4-6$ animals each experimental condition. * $P < 0.05$ vs. Basal; # $P < 0.05$ vs. Control rats.

Figure 6. Representative Western blot for COX-1, COX-2, eNOS, P-eNOS and iNOS expression in resistance mesenteric segments from control and cirrhotic rats. The figure is representative of four separate experiments. Lower panel shows densitometric analysis of the different blots. Results (Means \pm S.E.M.) are expressed as the ratio between the signal for the protein expression and the signal for α -actin. * $P < 0.05$ control vs. cirrhotic.

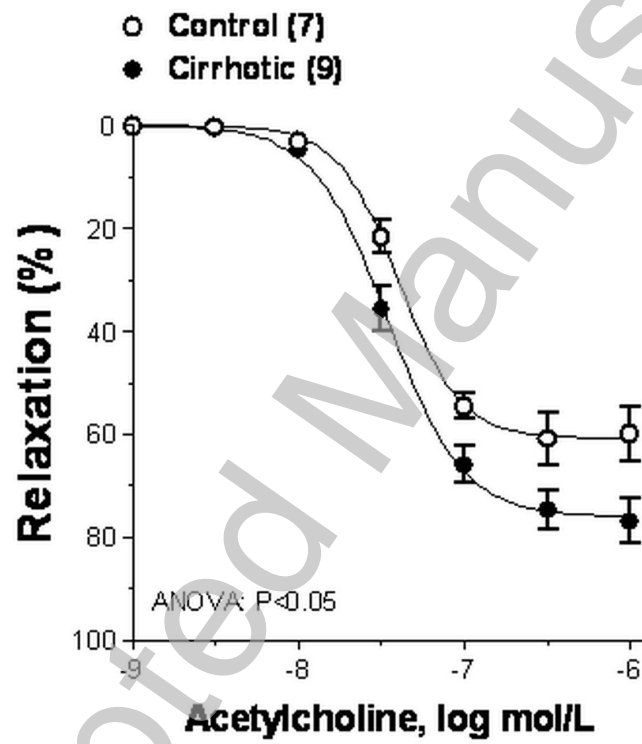


Figure 1

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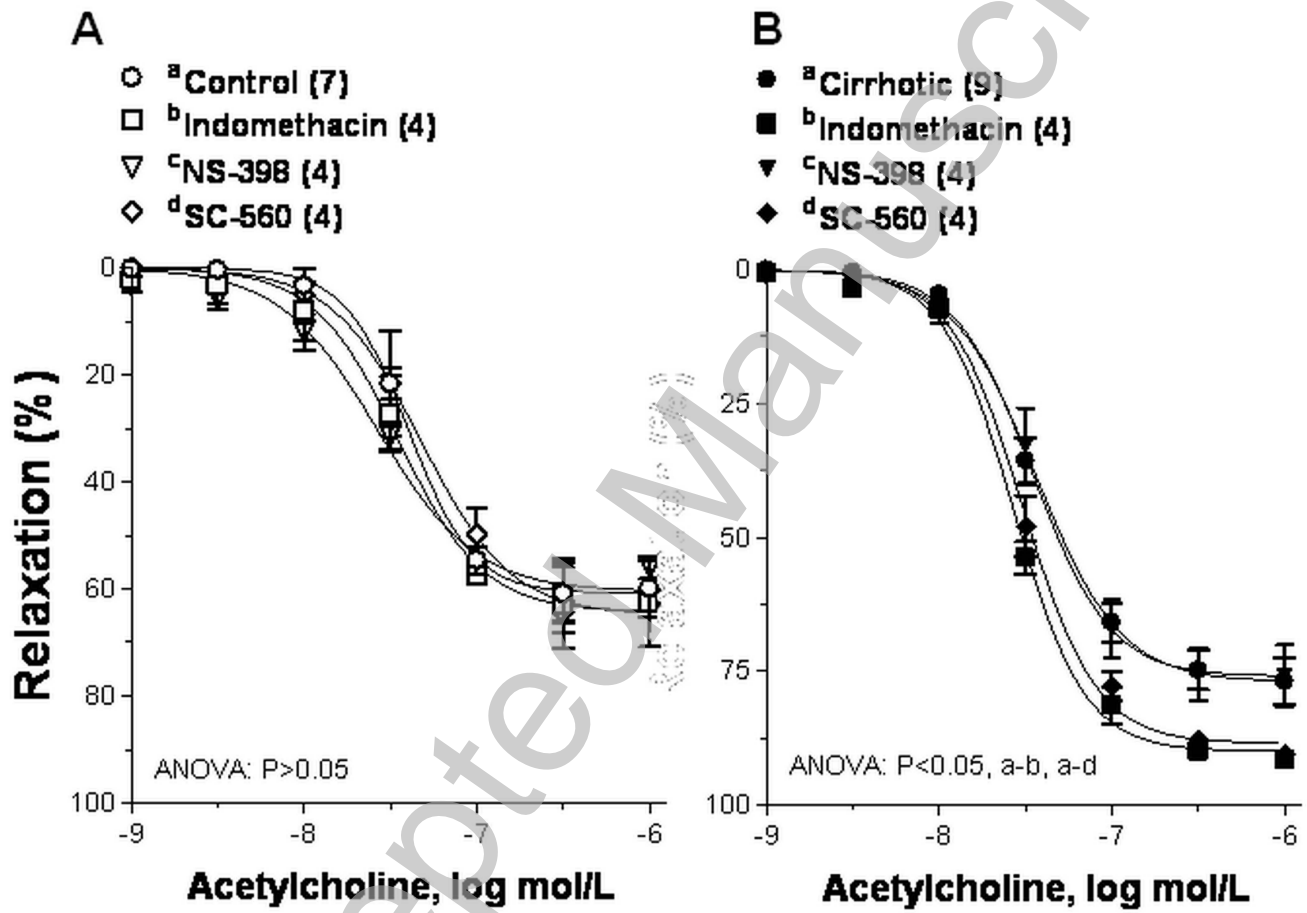


Figure 2

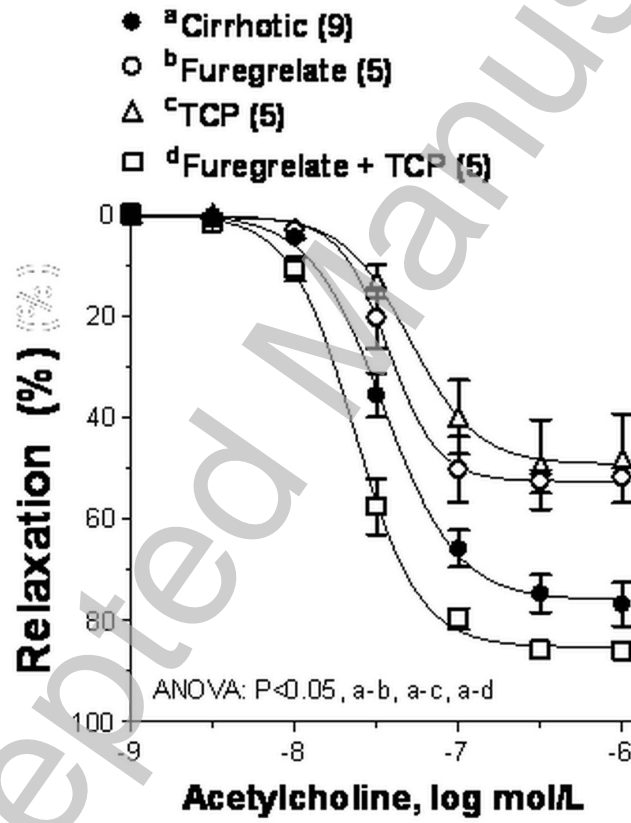


Figure 3

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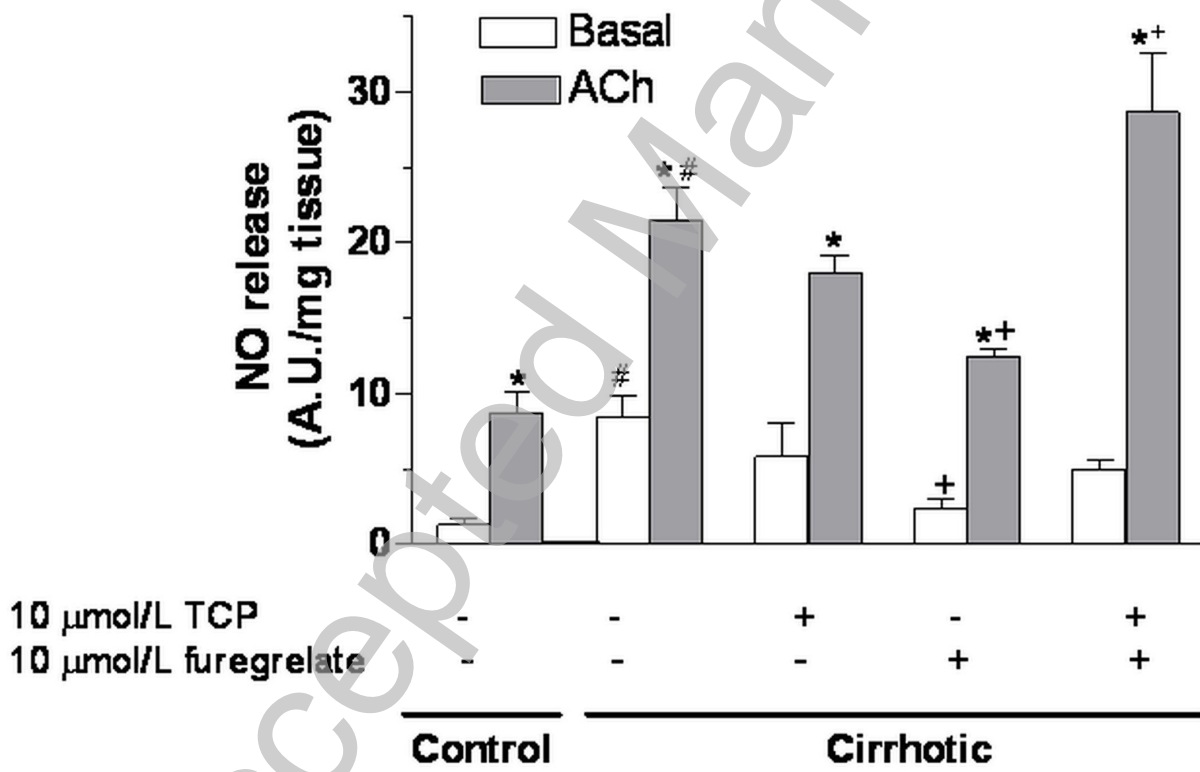


Figure 4

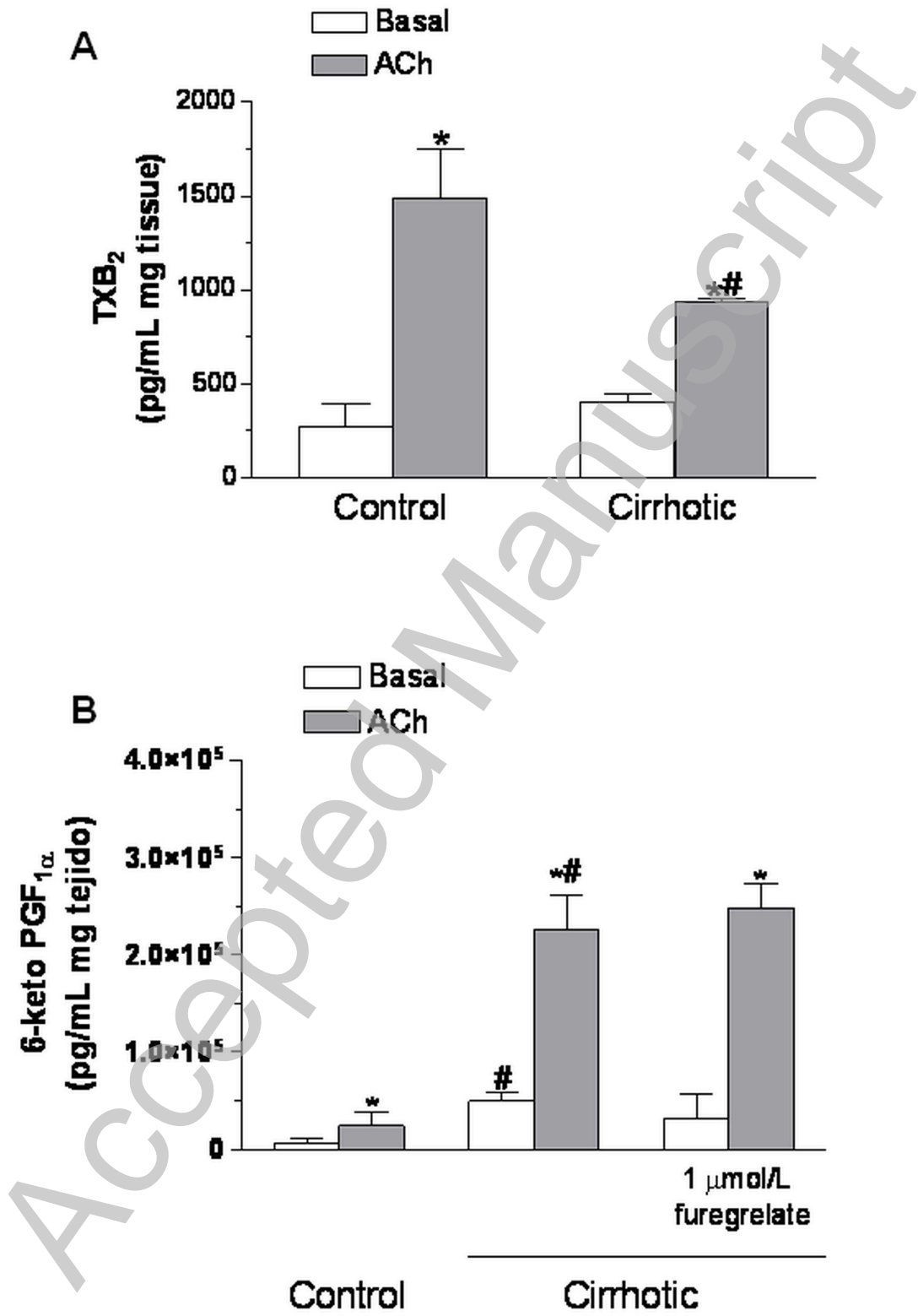


Figure 5

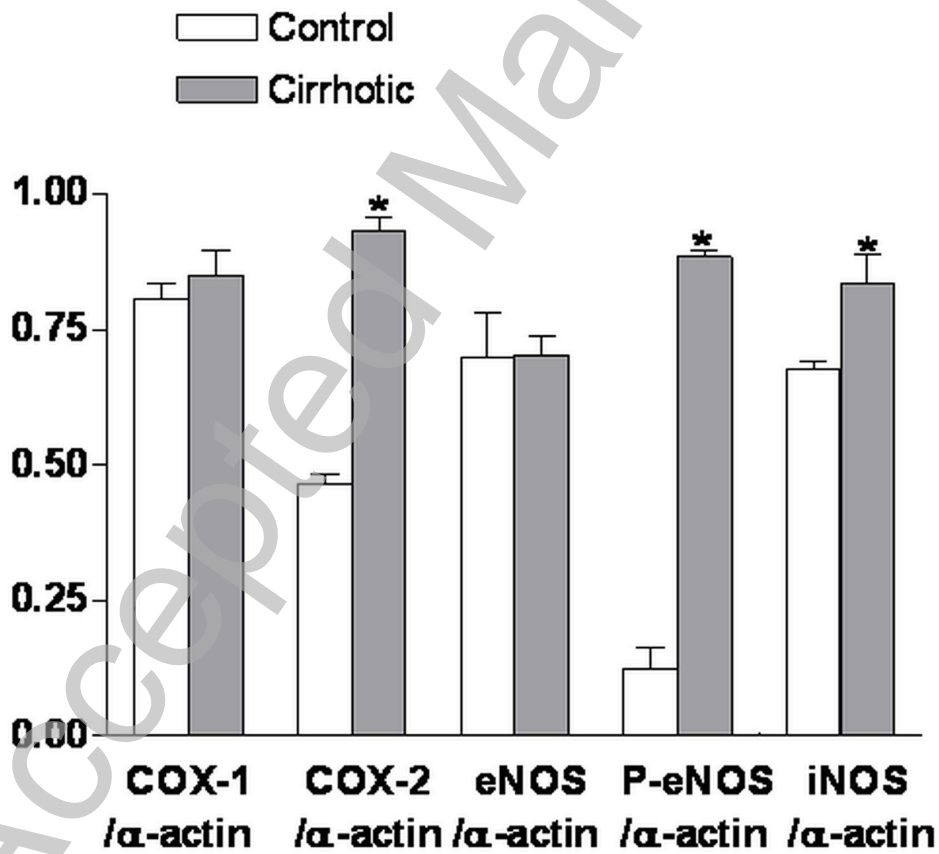
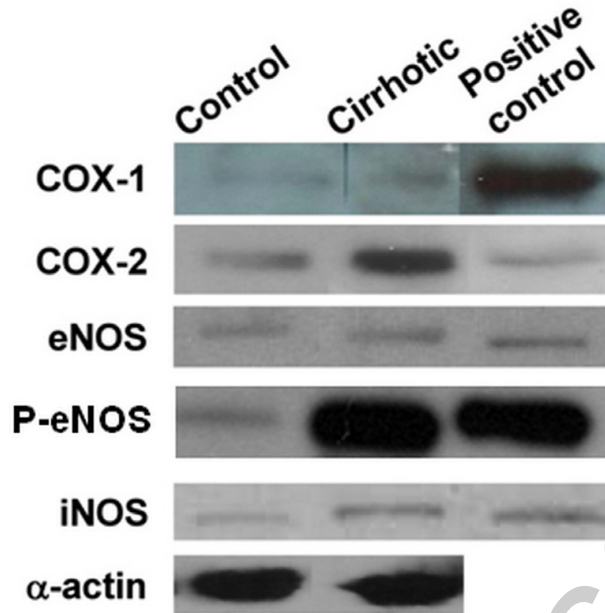


Figure 6