

# Amelioration of portal hypertension and the hyperdynamic circulatory syndrome in cirrhotic rats by neuropeptide Y via pronounced splanchnic vasoaction

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Revised 16 November 2010  
Accepted 27 November 2010  
Published Online First  
12 February 2011

## ABSTRACT

**Background** Splanchnic vasodilation triggers the development of the hyperdynamic circulatory syndrome in portal hypertension. Neuropeptide Y (NPY), a sympathetic co-transmitter of norepinephrine, improves contractility in mesenteric arteries of pre-hepatic portal hypertensive rats. Therefore, we investigated the effect of NPY on mesenteric arterial contractility in vitro and in vivo in cirrhotic ascitic rats, as well as the vasoactive pathways involved.

**Methods** All experiments were performed in CCl<sub>4</sub>-induced cirrhotic rats with ascites and compared to controls. In vivo haemodynamic characterisation was assessed before and after cumulative application of NPY i.v. using the microspheres technique. In vitro mesenteric arterial perfusion was used to analyse the effect of NPY on the response to  $\alpha_1$ -adrenergic, as well as nitric stimulation. The NPY effects on vasoactive pathways (RhoA/Rho-kinase and NOS/NO) were analysed by western blot in mesenteric arteries.

**Results** NPY decreased portal-venous blood flow and reduced portal pressure in cirrhotic rats, without changes in mean arterial pressure. This was accompanied by decreased cardiac output and normalised systemic vascular resistance in cirrhotic rats. By contrast, no significant splanchnic or systemic haemodynamic effect of NPY was seen in controls. NPY enhanced arterial contractility in cirrhotic but not in control rats. Furthermore, NO-mediated vasodilation was reduced to a greater extent than in controls. These findings were paralleled by an increased expression and activity of the constrictive Rho-kinase pathway and decreased activation of vasodilating NOS/NO signalling after NPY administration in mesenteric arteries.

**Conclusions** NPY exerts marked portal hypotensive effects and ameliorates the hyperdynamic circulation in cirrhotic ascitic rats. This is mediated mainly by a pronounced splanchnic vasoconstriction and reduction in splanchnic blood flow due to enhanced Rho-kinase expression and activity, as well as reduced NOS activation and NO effect.

## INTRODUCTION

Splanchnic arterial vasodilation is the pathophysiological hallmark in the development of the hyperdynamic circulatory syndrome and the associated haemodynamic derangements in portal hypertension.<sup>1</sup> This is due to (1) marked vascular overproduction of vasodilators, mainly nitric oxide

## Significance of this study

### What is already known on the subject?

- Portal hypertension and the hyperdynamic circulatory syndrome (HCS) are the cause of most complications limiting prognosis in advanced cirrhosis.
- Splanchnic vasodilation is the pathophysiological hallmark for the development of the HCS, aggravates portal hypertension and has been attributed mainly to vascular NO production and defective RhoA/Rho kinase signalling in the mesenteric circulation.
- Advanced cirrhosis associates with a marked stimulation of the sympathetic nervous system aiming to counterbalance arterial vasodilation.
- Neuropeptide Y (NPY) is a sympathetic neurotransmitter known to facilitate  $\alpha$ -adrenergic vasoconstriction. In mesenteric arteries this vasoaction is more pronounced in pre-hepatic portal hypertensive rats and, thus, corrects vascular hyporeactivity.

### What are the new findings?

- NPY is the first vasoactive substance exerting (in vitro and in vivo) higher vasoconstrictive action in the splanchnic circulation in portal hypertension than in healthy conditions making this peptide a superior candidate to counterbalance splanchnic vasodilation.
- Acute administration of NPY i.v. in vivo reduces portal hypertension by more than 20% and markedly attenuates the hyperdynamic circulation in decompensated cirrhotic rats.
- This most beneficial haemodynamic effect of NPY is due to its dual effect namely, inhibition of vascular NO overproduction as well as restoration of defective RhoA/Rho-kinase signalling in the mesenteric circulation during portal hypertension.

### How might it impact on clinical practice in the foreseeable future?

- NPY should be tested for its haemodynamic effects in cirrhotic patients potentially leading to a new therapeutic agent increasing the pharmacological armamentarium available for treatment of portal hypertension.

(NO); and (2) vascular hyporeactivity to endogenous vasoconstrictors.<sup>2</sup> Many studies report enhanced extrahepatic expression of NO synthases (inducible, endothelial and neuronal) associated with a marked vascular NO overproduction contributing to splanchnic vasodilation.<sup>2–7</sup> Moreover, splanchnic vascular hyporeactivity to vasoconstrictors is at least partially due to defective intracellular contractile signalling.<sup>8–10</sup> In humans and animals with cirrhosis, this splanchnic hyporeactivity is accompanied by a downregulated and defective RhoA/Rho-kinase pathway, which is crucial for maintaining vascular tone.<sup>10–12</sup> This splanchnic vasodilation sustains underfilling of the effective central blood volume and activation of vasoconstrictors. Therefore, improvement of splanchnic vasoconstriction is an important goal in therapy of portal hypertension and liver cirrhosis.

Stimulation of the sympathetic nervous system (SNS) might counterbalance this arterial vasodilation in portal hypertension.<sup>13</sup> Neuropeptide Y (NPY) is co-stored and co-released with norepinephrine from secretory vesicles of sympathetic nerve terminals.<sup>14</sup> NPY, predominantly located in sympathetic nerves of small arterioles is one regulator of vascular resistance.<sup>15</sup> It potentially facilitates adrenergic vasoconstriction by sensitising vascular smooth muscle for norepinephrine, even though the exact mechanism is unknown.<sup>16–18</sup> We have reported previously that NPY augments maximal  $\alpha_1$ -adrenergic vasoconstriction and thereby, corrects vascular hyporeactivity in a pre-hepatic model of portal hypertension.<sup>19–20</sup> The potentiative vasoconstrictive capacity of NPY was more pronounced in mesenteric arteries of portal hypertensive rats as compared to control rats. This indicates an important NPY-induced compensatory mechanism counterbalancing arterial vasodilatation and restoring the efficacy of endogenous catecholamines in the splanchnic circulation in portal hypertension. However, the haemodynamic effects of NPY in cirrhotic portal hypertension and the involved mechanisms remain unexplored. Therefore, we studied the effects of exogenous NPY in vivo as well as in vitro in the mesenteric circulation in cirrhotic rats with portal hypertension as well as involved molecular mechanisms.

## MATERIAL AND METHODS

### Animals

The investigation was performed in male Sprague–Dawley rats (Harlan Sprague Dawley Laboratories, Indianapolis, Indiana, USA), weighing 300–399 g. Rats were caged at a constant room temperature of 21°C, exposed to a 12:12 h light:dark cycle, and allowed free access to water and standard rat chow.

### CCl<sub>4</sub>-induced liver cirrhosis

Cirrhosis was induced in male pathogen-free CD rats (Charles River, 50–80 g initial weight) by inhalation of CCl<sub>4</sub> along with phenobarbital (0.35 g/l) in the drinking water, as previously described.<sup>21–22</sup> The CCl<sub>4</sub> administration was started three times a week over 1 min and increased every other week by 1 min to a maximum of 5 min, depending on change in body weight. After 12 to 16 weeks, this approach induces micronodular liver cirrhosis with ascites. Seven days prior to experimental procedures application of CCl<sub>4</sub> as well as phenobarbital was stopped. Only cirrhotic animals with decompensation of liver function and thus presence of ascites were used. Phenobarbital-treated age- and sex-matched rats were used as control group.

### In vivo haemodynamic studies

Median laparotomy was performed, and a PE-50 catheter was introduced into a small ileocaecal vein and advanced to the portal vein for the measurement of portal pressure (PP) as

previously described.<sup>12</sup> The left femoral artery was cannulated with PE-50 catheters for measurement of mean arterial pressure (MAP). Via the right carotid artery, another PE-50 catheter was advanced into the left ventricle under pulse curve control. This catheter was used for microsphere application. The catheters in the femoral artery and the portal vein were connected to a pressure transducer (Statham, Oxnard, California, USA) and continuously recorded (Powerlab Quadbridge and Powerlab 4/20; AD Instruments, Spechbach, Germany). The zero point was 1 cm above the operating table. After insertion of all catheters, rats were allowed to stabilise haemodynamically for 30 min. NPY was administered intravenously as bolus (0.1 ml) in a cumulative manner in doses ranging from 0.005 nmol up to 100 nmol and MAP and PP were monitored continuously for a further 20 min, followed by application of the microsphere technique.

### Microsphere technique

Cardiac output was measured using the coloured microsphere method as previously described.<sup>12–23</sup> The coloured microsphere technique was validated by the more frequently used radioactive microsphere method. It has the advantage of being non-radioactive. A reference sample was obtained for 1 min at a rate of 0.65 ml/min, using a continuous withdrawal pump (Hugo Sachs Elektronik, March-Hugstetten, Germany). About 300 000 yellow microspheres (15  $\mu$ m diameter; Triton Technologies, San Diego, California, USA) were suspended in 0.3 ml saline containing 0.05% Tween and injected in the left ventricle 10 s after the withdrawal pump had been started. Mesenteric portal-systemic shunt volume was estimated after injection of 150 000 blue microspheres in 0.3 ml saline containing 0.05% Tween in an ileocaecal vein within 30 s.

The blood reference probe was digested by addition of 3.8 ml 5.3 M KOH and 0.5 ml Tween 80 and subsequent boiling for 1 h. The digested tissues and blood samples were vortexed and filtered using Whatman Nucleopore filters (Whatman International, Maidstone, UK). The colour of the filtered microspheres was dissolved in 0.2 ml dimethyl formamide, and the absorption was measured using spectrophotometry. Thereafter, cardiac output and organ blood flow was calculated using software obtained from Triton Technologies and expressed per 100 g body weight. Splanchnic perfusion pressure was defined as MAP minus PP. Splanchnic vascular resistance was calculated from the ratio between splanchnic perfusion pressure and the measured splanchnic blood flow, without including hepatic arterial flow. Mesenteric portal-systemic shuntflow was measured as the fraction of blue microspheres in the lung from total blue microspheres injected in an ileocaecal vein. Hepatic portal-vascular resistance was estimated as PP divided by the sum of gastrointestinal and splenic perfusion minus mesenteric portal-systemic shuntflow. Systemic vascular resistance (SVR) was calculated as the ratio between MAP and cardiac output. Vascular resistance of specific organs (kidney, spleen, liver, stomach–gut) was calculated as the ratio between MAP and organ blood flow (table 1).

### In vitro perfusion

The in vitro perfusion system used was a partial modification of that originally described by McGregor and used extensively in previous studies in our laboratory.<sup>24–25</sup> Briefly, the superior mesenteric artery (SMA) was cannulated with a PE-60 catheter and gently perfused with 15 ml warm Krebs solution to eliminate blood. After isolating the SMA with its mesentery, the gut was cut off close to its mesenteric border. The arterial vasculature was then transferred to a 37°C water-jacketed container and

**Table 1** In vivo haemodynamic data before and after administration of neuropeptide Y (NPY)

Parameter	Control			Cirrhosis NPY
	Basal	NPY	Basal	
Mean arterial pressure (mmHg)	120.1±5	118.8±9	101.4±7*	99.9±9*
HR (/min)	227.5±22.7	257±30	362.7±25.3**	323±36** ††
Portal pressure (mmHg)	7.5±0.6	7.7±0.5	14.1±0.8***	10.9±0.5* †††
CO (ml/min/100 g)	18.6±2.3	18.9±2.3	30.5±4.6*	23.7±3.8** ††
Systemic vascular resistance (mmHg/min/100 g/ml)	6.4±0.5	6.2±0.5	4.2±0.6*	6.3±1.5†
SpBF (ml/min/100 g)	1.9±0.5	1.8±0.4	5.6±0.8**	4.0±0.5** †
Splanchnic vascular resistance (mmHg/min/100 g/ml)	62.1±10.9	72.7±18	18.8±4.1**	26.6±9** †
HABF (ml/min/100 g)	2.2±0.2	1.8±0.4	3.5±0.6*	2.8±0.5
HPVR (mmHg/min/100 g/ml)	1.8±0.2	2.2±0.4	2.3±0.4	1.5±0.2
Renal blood flow (ml/min/100 g)	1.36±0.38	0.91±0.36†	1.89±0.69	1.85±0.70*
Renal vascular resistance (mmHg/min/100 g/ml)	0.06±0.02	0.06±0.01	0.07±0.02	0.09±0.07
Mes-portal shunt‡ (ml/min/100 g)	—	—	0.45±1.1	0.39±0.4

† Obtained in a separate set of experiments versus basal: \*p<0.05; \*\*p<0.01; \*\*\*p<0.001/versus basal: †p<0.05; ††p<0.01; †††p<0.001.

CO, cardiac output; HABF, hepatic arterial blood flow; HPVR, hepatic portal vascular resistance; HR, heart rate; SpBF, Splanchnic blood flow.

perfused with oxygenated 37°C Krebs solution (95% O<sub>2</sub>, 5% CO<sub>2</sub>) using a roller pump (Isamtec, IPC 8-channel; Glattburg, Zürich, Switzerland). The Krebs solution had the following composition (in mmol/l): NaCl, 118; KCl, 4.7; KH<sub>2</sub>PO<sub>4</sub>, 1.2; MgSO<sub>4</sub>, 1.2; CaCl<sub>2</sub>, 2.5; NaHCO<sub>3</sub>, 25; disodium EDTA, 0.026; and glucose, 11.0; pH 7.4. The effluent of the perfused tissue was continuously removed from the perfusing chamber. The perfusion pressure was measured with a P-23-Db strain gauge transducer (Statham) on a side arm just before the perfusing cannula and continuously recorded (Powerlab Quadbridge and Powerlab 4/20; AD Instruments).

Where indicated, endothelial denudation of the mesenteric vasculature was performed by a combined treatment of cholic acid (sodium salt) and distilled water as has been used before.<sup>26</sup> In brief, after cannulation of the SMA and gentle flushing with 10 ml of warmed Krebs solution to eliminate blood, perfusion with cholic acid (0.5%/1.5 ml for 10 s) followed by 15 ml of Krebs solution (to eliminate cholic acid) was performed. The preparation was then transferred to the 37°C water-jacketed container and perfused with oxygenated 37°C Krebs solution (4 ml/min) for 10 min. After the mesenteric vasculature was relaxed, 37°C warmed distilled water was perfused for 10 min before starting Krebs perfusion again. After an equilibration period of 45 min, experimental perfusion protocols were performed (figure 1). At the end of each experiment we assessed whether the vessel was completely de-endothelialised and whether the smooth muscle function was maintained. The mesenteric preparation was kept precontracted with methoxamine (MT; alpha-1-agonist:

100 µM) and dose-dependent vasorelaxation to the endothelium-dependent vasodilator acetylcholine (ACh: 10<sup>-8</sup> to 10<sup>-6</sup> g, bolus of 0.1 ml) and the endothelium-independent vasodilator sodium nitroprusside (SNP: 10<sup>-6</sup> to 10<sup>-5</sup> g, bolus of 0.1 ml) was tested. Only experiments with ACh- and SNP-induced relaxation being less/more than 15%/60% were accounted as valid.

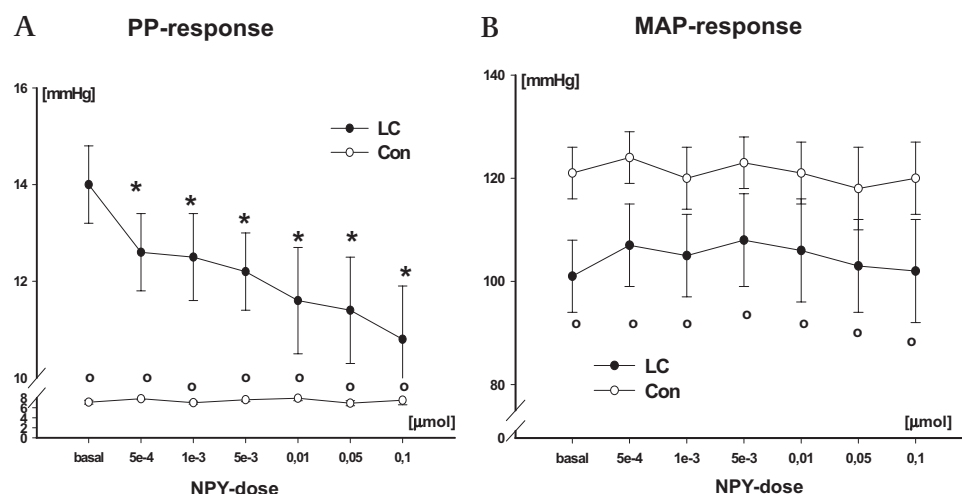
### In vitro study protocol

Baseline perfusion at 4 ml/min was established for 30 min before different study protocols were performed. Where indicated, two perfusion cycles following the same pharmacological test were done. After completion of the first perfusion cycle and a wash-out period of 45 min, NPY (50 nM) was added and a second perfusion cycle was initiated after 10 min of incubation time. It is important to stress that in preliminary studies, 50 nM NPY concentration was found to have no intrinsic direct vasoconstrictive action and did not potentiate vasoconstriction at low and medium vascular tone. NPY was present at the same molar concentration in the perfusion system throughout the second perfusion cycle. As shown previously the perfusion system showed stable basal perfusion conditions and unchanged pressor responsiveness for the time period necessary for this experimental protocol.

### Protocol I: NPY-evoked vasoconstriction in vitro

This protocol was performed in de-endothelialised mesenteric preparations. Considering the well-known enhanced release of endothelium-derived vasodilators mainly nitric oxide, in portal

**Figure 1** Effect of neuropeptide Y (NPY) on portal pressure (A) and mean arterial pressure (B) in control (n=7) and cirrhotic ascitic rats (LC, n=8). \*: p<0.05 versus basal; o: p<0.05 versus control rats. LC, liver cirrhosis; PP, portal pressure.



hypertension in the splanchnic circulation by this approach we avoided potential differences in shear-mediated release of endothelium-derived vasodilators, between the study groups. In order to test a direct vasoconstrictive effect, NPY was administered as repeated boluses (0.1 ml) at increasing doses (0.1 nM to 10  $\mu$ M) in intervals of 2–4 min in the absence of vasoconstrictors (at baseline conditions). In a different protocol, mesenteric tissue preparations were precontracted with MT and after reaching a stable precontraction plateau, cumulative dose–response curves to NPY, administered in the form of repeated boluses at increasing doses (see above) were obtained. In order to test for the differences in NPY-induced vasoconstriction dependent on the degree of  $\alpha_1$ -adrenergic stimulation increasing levels of  $\alpha_1$ -adrenergic precontraction using 0.3, 1, 3 and 10  $\mu$ M of MT were applied.

#### Protocol II: NPY-induced facilitation of $\alpha_1$ -adrenergic vasoconstriction in vitro

This protocol was performed in intact mesenteric preparations. Non-cumulative dose-response curves for MT (0.3–300  $\mu$ M) were performed by continuous infusion for 2 min for each dose administered (first and second perfusion cycle).

#### Protocol III: NPY effect on eNOS-dependent vasorelaxation in vitro

Intact mesenteric vessel preparations were pre-constricted with MT ( $EC_{50}$ ). After achieving a stable pre-contraction level, the preparation was stimulated by increasing doses of ACh ( $10^{-8}$  to  $10^{-5}$  M). ACh was given in non-cumulative manner administered as repeated boli (0.1 ml) in the first and second perfusion cycle.

#### Protocol IV: NPY effect on nitrgic vasorelaxation in vitro

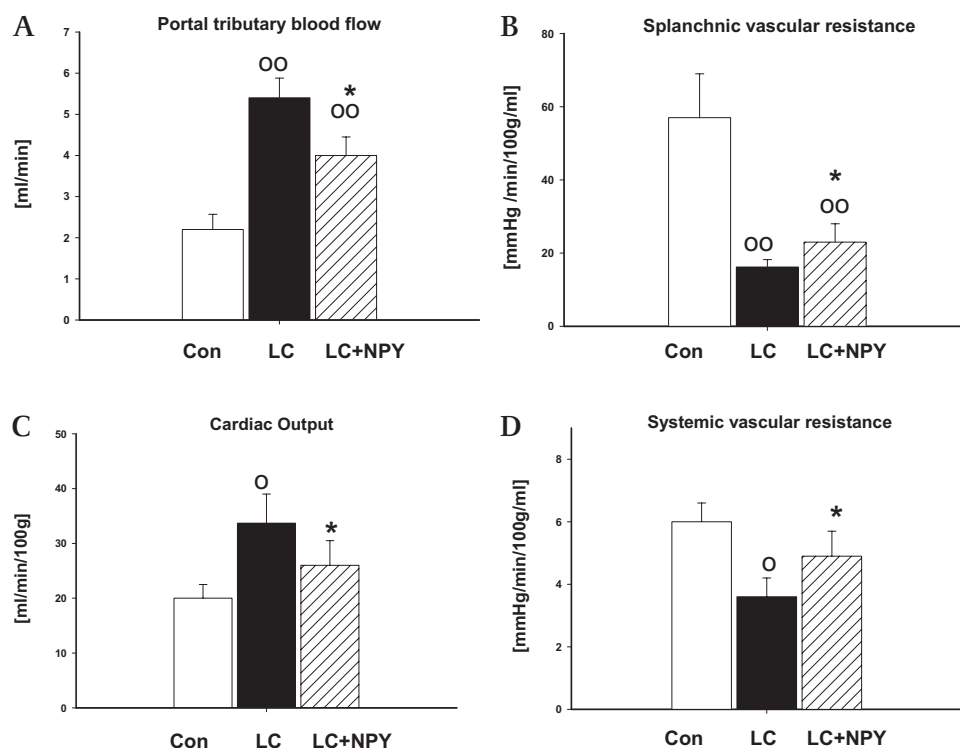
Periarterial nerve stimulation (PNS) was used to investigate non-adrenergic and non-cholinergic vasorelaxation in de-endothelialised mesenteric arteries. Two platinum electrodes, one placed around the SMA and the other shaped as a wire grid the tissue was resting on, were used for transmural electrical field stimulation. The nerves of the preparation were stimulated by means

of an electronic stimulator (I-ZQ4v; Hugo Sachs Electronics, Hugstetten, Germany), delivering single square-wave pulses (2 ms) at 45 V with a train duration of 30 s and frequencies of 2–12 Hz. In order to evaluate vasodilatory responsiveness vessels were precontracted submaximally ( $EC_{80}$ ) using norepinephrine (NE  $10^{-5}$  M) before applying PNS. nNOS-mediated vasorelaxation is known to be non-adrenergic and non-cholinergic in origin. Therefore, guanethidine ( $5 \times 10^{-5}$  M), atropin ( $10^{-9}$  M) and timolol ( $10^{-9}$  M) were added from the beginning in order to deplete endogenous norepinephrine stores and to prevent its uptake as well as to avoid cholinergic stimulation. When a stable precontraction level was achieved, PNS was applied in a non-cumulative fashion. Sufficient time was allowed between each stimulation train for the perfusion pressure to return to a stable level, usually within 5–10 min. PNS responses are expressed as percentage change of the pre-contraction level present before PNS. Nitrgic vasodilation is known to be independent of prostaglandin synthesis<sup>27</sup> and it is not subject of pre-junctional inhibition by  $\alpha_2$ -adrenoreceptors.<sup>27</sup>

#### Western blotting

In five control as well as five cirrhotic CCl<sub>4</sub> rats, 50 nM NPY was administrated through the femoral vein. After 1 h, animals were sacrificed for tissue harvesting to study the RhoA/Rho-kinase and nitric oxide synthase/protein kinase G (NOS/PKG) activity. Respectively, control and cirrhotic CCl<sub>4</sub> rats after administration of vehicle in the femoral vein served as controls. Samples of shock-frozen mesenteric arteries were homogenised in a buffer containing 25 mM Tris/HCl, 5 mM ethylenediamine tetraacetic acid, 10  $\mu$ M phenylmethanesulfonyl fluoride, 1 mM benzamidine, and 10  $\mu$ g/ml leupeptin.<sup>12 28</sup> Samples were diluted with sample buffer. Protein determination of the homogenates was performed with the Dc-Assay kit (Biorad, Munich, Germany). Samples (20  $\mu$ g of protein/lane) were subjected to SDS-PAGE (15% gels for RhoA; 8% gels for Rho-kinase, iNOS, eNOS and p-eNOS; 10% gels for moesin, p-moesin, VASP and p-VASP), and proteins were blotted on nitrocellulose membranes. The

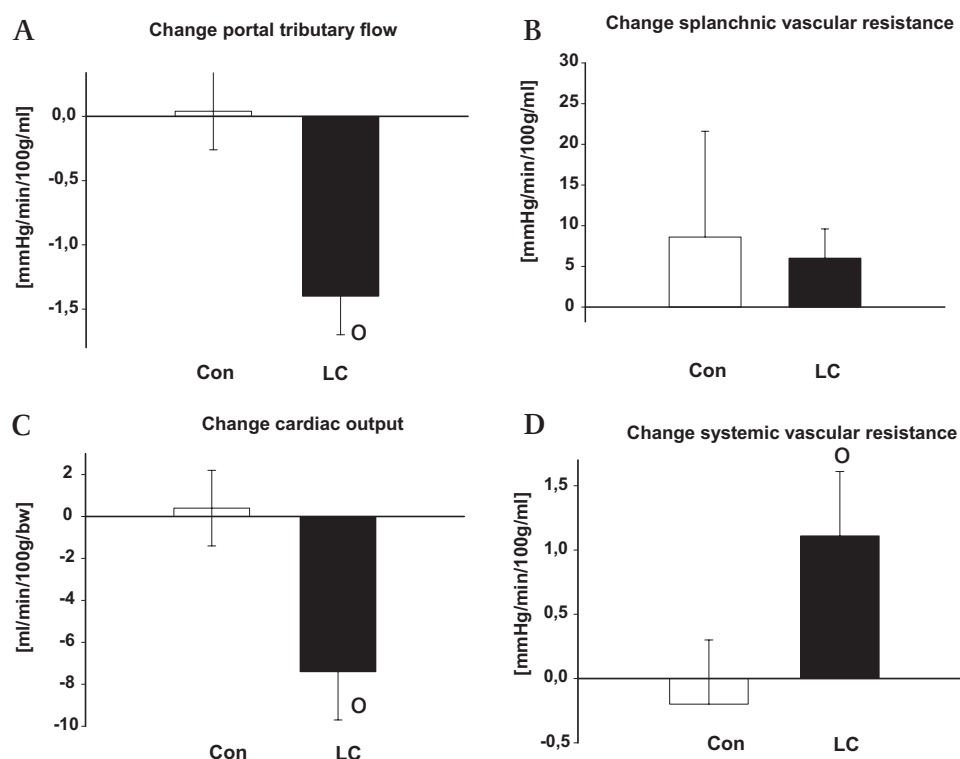
**Figure 2** Portal tributary flow (A), splanchnic vascular resistance (B), cardiac output (C) and systemic vascular resistance (D) in control (n=7) and cirrhotic ascitic rats (LC, n=8) after acute intravenous administration of neuropeptide Y (NPY). \*:  $p < 0.05$  versus LC, o:  $p < 0.05$  and oo:  $p < 0.01$  versus control. LC, liver cirrhosis.





## Hepatology

**Figure 3** Change in portal tributary flow (A), splanchnic vascular resistance (B), cardiac output (C) and systemic vascular resistance (D) induced by neuropeptide Y (NPY) in control (n=7) and cirrhotic ascitic rats (LC, n=8). o:  $p < 0.05$  versus control. LC, liver cirrhosis.



membranes were blocked, incubated with primary antibodies: RhoA 119, Rock-2 H-85, NOS3, moesin, p-moesin, VASP and GAPDH from Santa Cruz Biotechnology (Santa Cruz, California, USA); iNOS (ab49999) from Abcam (Cambridge, UK); p-eNOS (Ser 1177) from Cell Signaling (Boston, Massachusetts, USA); p-VASP clone 16C2 from Calbiochem (San Diego, California, USA). Thereafter the membranes were incubated with the corresponding secondary peroxidase-coupled antibodies (Calbiochem). Blots were developed with enhanced chemiluminescence (ECL, Amersham, UK). Intensities of the resulting bands on each blot were compared densitometrically with a FLA-3000 phosphoimager (Fuji-Film, Düsseldorf, Germany).

### Assessment of PKG and Rho kinase activity

PKG activity was assessed as phosphorylation of the endogenous PKG substrate, VASP, at Ser-239. The phosphorylation state of VASP serves as a marker for PKG activity. Rho-kinase activity was assessed as phosphorylation of the endogenous Rho kinase substrate, moesin, at thr-558. This was done by western blot analysis using site- and phosphospecific antibodies.<sup>12 28</sup>

### Statistical analysis

Results were expressed as mean  $\pm$  SE. Statistical analysis was performed using ANOVA (two-way, with repeated measurements) or the paired and unpaired Student *t* test if appropriate. The statistical significance level was  $p < 0.05$ .

## RESULTS

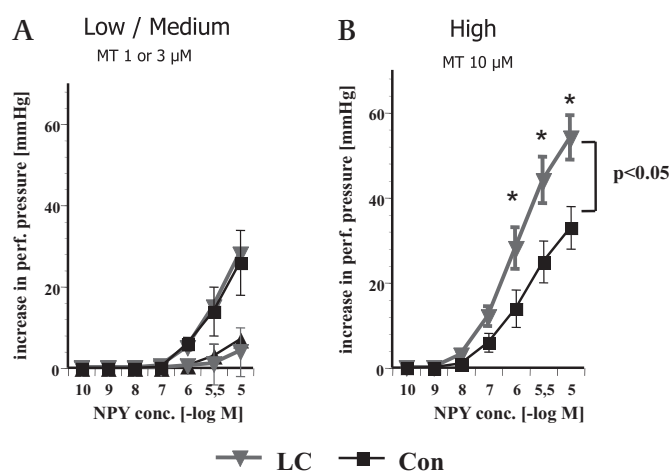
### Animals

There were no significant differences in body weight in the experimental groups ( $446 \pm 23$  g for cirrhotic and  $380 \pm 15$  g for control rats, respectively). Cirrhotic rats showed elevated spleen weights, expressed as percentage of body weight ( $3.90 \pm 0.24$  g/kg b.w. vs sham:  $2.44 \pm 0.23$  g/kg b.w.,  $p < 0.0001$ ).

### In vivo haemodynamic studies

As expected, cirrhotic rats presented with markedly increased PP when compared to control rats (figure 1A). Intravenous NPY

application caused a dose-dependent decrease in PP in cirrhotic rats without significantly altering PP in control rats (figure 1A). Basal MAP was significantly lower in cirrhotic rats as compared to control rats (figure 1B). NPY had no significant effect on MAP at any dose given in both groups. Cirrhotic rats exhibited a marked hyperdynamic circulation with significantly enhanced cardiac output and portal tributary blood flow associated with a markedly decreased systemic and splanchnic vascular resistance (figure 2). Intravenous application of NPY elicited a significant drop in portal tributary blood flow (figure 2A) and cardiac output (figure 2C) in conjunction with a marked increase in splanchnic



**Figure 4** Neuropeptide Y (NPY)-evoked potentiation of  $\alpha_1$ -adrenergic vasoconstriction in dependency on the level of sympathoadrenergic activity. Dose-response curves of NPY in mesenteric vasculature after removal of the endothelium and pre-constricted with different doses of methoxamine: A: low (1  $\mu$ M) and medium dose (3  $\mu$ M) and B: high dose (10  $\mu$ M) of MT used for pre-constriction. Measurements represent at least three experiments for each study group and dose of MT used. \* $p < 0.05$  versus control. MT, methoxamine.

and systemic vascular resistance in cirrhotic rats (figure 2B,D), without hepatic or renal effects (table 1). In contrast, in control rats, NPY only caused a slight but not significant amelioration in portal tributary blood flow and no change in cardiac output and systemic haemodynamics (figure 3). NPY did abrogate the basal difference in cardiac output and systemic vascular resistance between the study groups (figure 3). Changes in portal tributary blood flow, cardiac output and systemic vascular resistance induced by NPY were significantly greater in cirrhotic rats as compared to control rats (figure 3). This strongly indicates a more pronounced vasoaction of NPY in the splanchnic circulation in cirrhotic rats as compared to control rats (figure 1–3).

### In vitro perfusion experiments

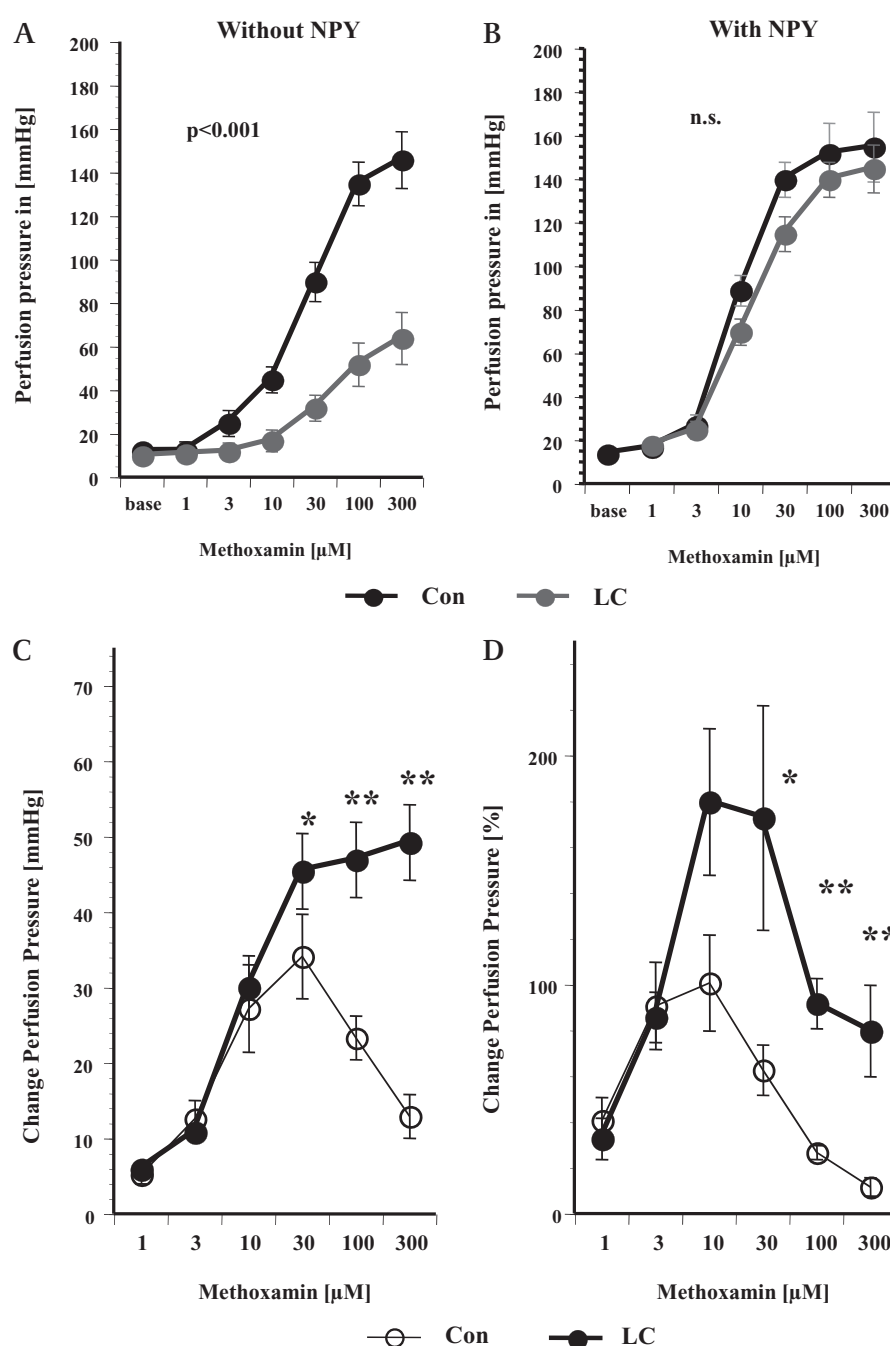
Baseline perfusion pressures for all study protocols were significantly lower in cirrhotic rats as compared to sham rats evidencing the presence of arterial vasodilation ( $7.2 \pm 1$  mm Hg vs

$11.4 \pm 0.7$  mm Hg,  $p < 0.0001$ ). Removal of the endothelium significantly increased baseline pressures in both groups, rendering baseline conditions no more significantly different between cirrhotic and control rats ( $14.4 \pm 1.8$  mm Hg vs  $16.5 \pm 1.1$  mm Hg).

### NPY-evoked vasoconstriction in vitro in de-endothelialised mesenteric arteries

As reported earlier NPY did not induce any significant effect in the absence of vasoconstrictors demonstrating a clear lack of direct vasoconstrictive action (data not shown). However, after MT-induced precontraction NPY enhanced  $\alpha_1$ -adrenergic vasoconstriction in a dose-dependent manner (figure 4). Since experiments were performed in de-endothelialised mesenteric preparations this effect confirms the endothelium-independent mode of action of NPY on vascular smooth muscle. The various pre-contraction levels in mesenteric preparations used were not significantly different between groups (data not shown). The

**Figure 5** Vascular responsiveness to  $\alpha_1$ -adrenergic stimulation in mesenteric arteries in cirrhotic ascitic and sham rats before and after neuropeptide Y (NPY) incubation. A marked vascular hyporesponsiveness to methoxamine can be appreciated between LC (n=6) and control animals (n=6) in the absence of NPY (A). After incubation with NPY vascular sensitivity and maximal contractility were no longer different between study groups (B). Changes in pressure response to methoxamine induced by NPY presented as absolute change in perfusion pressures as compared to values obtained during the first perfusion cycle (C). Changes in pressure response presented as percentage of perfusion pressure obtained during first perfusion cycle (D). n=6 for LC and control animals. \* $p < 0.01$ ; \*\* $p < 0.001$  versus sham animals. LC, liver cirrhosis.

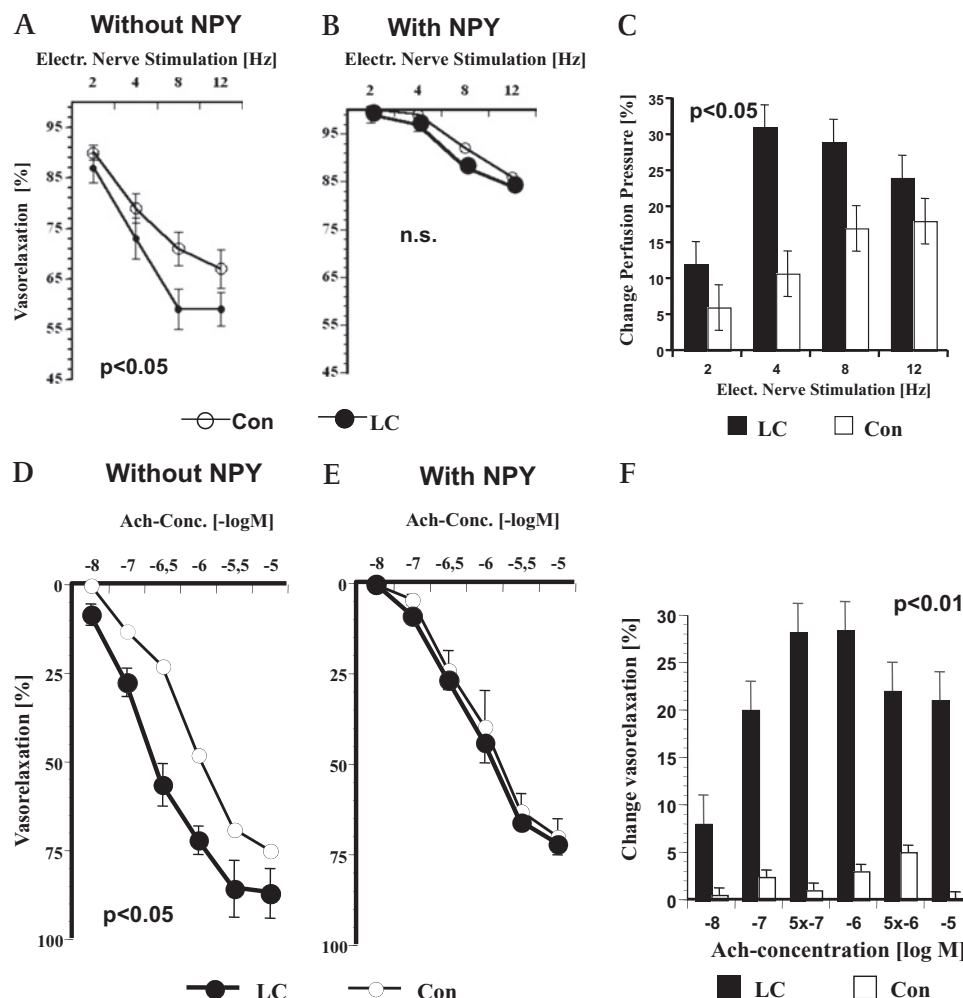


effect of NPY on  $\alpha_1$ -adrenergic vasoconstriction did increase in magnitude with increasing levels of  $\alpha_1$ -adrenergic pre-constriction. For instance, NPY-induced increases in perfusion pressure were significantly greater when administered to vessels pre-constricted at 3  $\mu\text{M}$  as compared to 1  $\mu\text{M}$  and the latter effect being greater than the one observed at 0.3  $\mu\text{M}$  (figure 4A;  $p < 0.001$  by ANOVA, respectively). At low and medium  $\alpha_1$ -adrenergic pre-constriction (MT: 0.3; 1 and 3  $\mu\text{M}$ , respectively) cumulative dose-response curves for NPY were not significantly different between cirrhotic and control rats (figure 4A). In contrast, after adrenergic precontraction using high doses of MT (10  $\mu\text{M}$ ) NPY-evoked vasoconstriction of de-endothelialised arterial mesenteric bed was significantly more marked in cirrhotic than in control animals (figure 4B).

### Effect of NPY on $\alpha_1$ -adrenergic vasoconstriction in vitro in mesenteric arteries with intact endothelium

Cirrhotic ascitic rats presented the well-known vascular hyporeactivity of intact mesenteric arteries to  $\alpha_1$ -adrenergic stimulation by MT (figure 5A). It is important to note that vascular sensitivity was not markedly decreased in cirrhotic rats whereas vascular contractility was markedly decreased (figure 5). After incubation with NPY, and in the presence of NPY, the vascular response to MT was no longer significantly different between the study groups (figure 5B). The change in vascular sensitivity, namely a left-ward shift of the MT dose-response curve, was similar in cirrhotic and healthy control rats ( $\text{EC}_{50}$ :  $9.5 \pm 1.7$  vs  $20.7 \pm 1.9$   $\mu\text{M}$  and  $14.1 \pm 2.0$  vs  $27.3 \pm 4.7$   $\mu\text{M}$ , respectively).

**Figure 6** Effect of neuropeptide Y (NPY) on nitrgic vasodilation in de-endothelialised mesenteric arteries, as well as its effect on eNOS-dependent vasodilation in mesenteric arteries with intact endothelium of cirrhotic ascitic and sham rats. Absolute pressure decrease induced by perivascular electrical nerve stimulation before (A) and after (B) NPY incubation. After NPY addition no more significant differences in frequency-dependent neural vasorelaxation was observed between study groups. Changes in nitrgic vasodilation induced by NPY (C) reveal a more pronounced inhibitory action of NPY in cirrhotic rats (LC group:  $n=6$ , sham group:  $n=6$ ). Absolute pressure decrease induced by acetylcholine before (D) and after (E) NPY incubation. After NPY addition no more significant differences in eNOS-dependent vasorelaxation was observed between study groups. Changes in eNOS-dependent vasodilation induced by NPY (F) reveal a more pronounced inhibitory action of NPY in cirrhotic rats (LC group:  $n=4$ , sham group:  $n=4$ ). eNOS, endothelial nitric oxide synthase; LC, liver cirrhosis.



However, the efficacy of MT at  $E_{\text{max}}$  was markedly augmented in cirrhotic rats but was not significantly altered in sham animals. Therefore, the NPY effect on vascular contractility was markedly more pronounced in cirrhotic rats. In fact, the improvement in pressure response induced by NPY at high concentrations of MT ( $> 10$   $\mu\text{mol/l}$ ) expressed as absolute changes in perfusion pressure was significantly higher in cirrhotic animals than in control rats (figure 5C). Expressing data as per cent change from the response obtained in the first perfusion cycle (in the absence of NPY) induced by NPY cirrhotic rats responded significantly more than control rats at high ( $> 10$   $\mu\text{mol/l}$ ) but not at low doses of the  $\alpha_1$ -adrenergic agonist (figure 5D).

### NPY-induced inhibition of vasorelaxation

PNS induced vasorelaxation in de-endothelialised mesenteric arteries. (figure 6). Vasodilator response to acetylcholine at the highest dose used was  $9.5 \pm 5\%$  and  $9.0 \pm 6\%$  in cirrhotic and control rats, respectively (NS) demonstrating a sufficient de-endothelialisation. Vasodilator response to sodium nitroprusside at the highest used dose was  $75 \pm 7\%$  and  $78 \pm 9\%$  for cirrhotic and control rats (NS) demonstrating the functional integrity of the vascular smooth muscle in the vascular bed studied. NE-induced pre-constriction levels were similar in both study groups ( $86 \pm 6$  vs  $91 \pm 5$  mm Hg) indicating similar vascular tone before neuronal stimulation. Incubation with NPY did not affect baseline or pre-constriction levels in either group. All vessels responded to PNS with a frequency-dependent decrease in perfusion pressure (figure 6A). This neurally mediated

vasodilatory response was significantly more pronounced in cirrhotic as compared to sham rats (figure 6A). NPY abolished this difference in PNS-induced vasorelaxation between the study groups (figure 6B). When expressing PNS-induced vasorelaxation as per cent change from pre-constriction level a markedly increased response was observed in vessel preparation from cirrhotic rats as compared to control rats (figure 6C).

Acetylcholine-induced vasorelaxation represents eNOS-dependent vasodilation and was enhanced in cirrhotic ascitic rats as compared to control rats (figure 6D). NPY inhibited eNOS-dependent vasorelaxation and thereby, abolished the difference between both study groups (figure 6E). In fact, the per cent change of vasorelaxation induced by NPY was more pronounced in cirrhotic rats as compared to control animals (figure 6F).

#### Effect of NPY on expression and activity of vasoactive proteins in mesenteric arteries of control rats

NPY administration elicited no significant changes in mesenteric expression of RhoA and total moesin in controls (data not shown), while an important increase in expression of Rho-kinase was observed (figure 7A). The activity of Rho-kinase was three times elevated by NPY, as measured by phosphorylation of its substrate moesin (figure 7A).

This activation of the contracting signalling RhoA/Rho-kinase was paralleled by a reduced NO effect at the same extent. This

was analysed as a 60% drop in activity of the NO effector PKG, measured as the phosphorylation of its substrate VASP (figure 7B). This was due to inhibition of eNOS activation, measured as its phosphorylation, as well as decreased expression of iNOS (figure 7B).

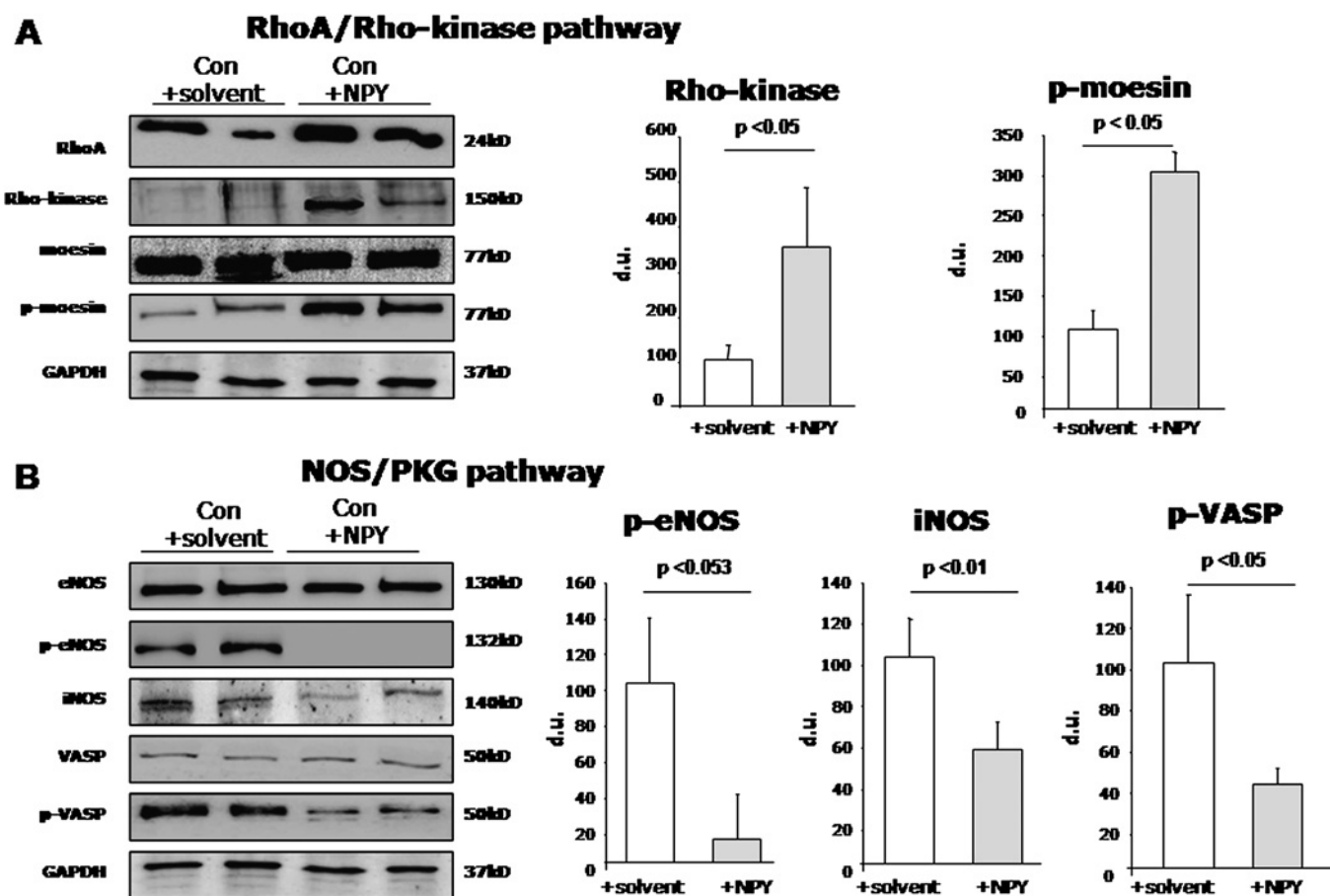
#### Effect of NPY on expression and activity of vasoactive proteins in mesenteric arteries of cirrhotic rats

Similarly to controls NPY administration elicited no significant changes in mesenteric expression of RhoA and total moesin in cirrhotics, while an important increase in expression of Rho-kinase was observed (figure 8A). The activity of Rho-kinase in cirrhotic mesenteric arteries was elevated by NPY by a factor of 7, as measured by phosphorylation of its substrate moesin (figure 8A).

The enhanced activation of eNOS and expression of iNOS were decreased by approximately 30% as shown in figure 8B. NPY administration decreased through this the NO effect by 50%, analysed as the PKG activity phosphorylating its substrate VASP (figure 8B).

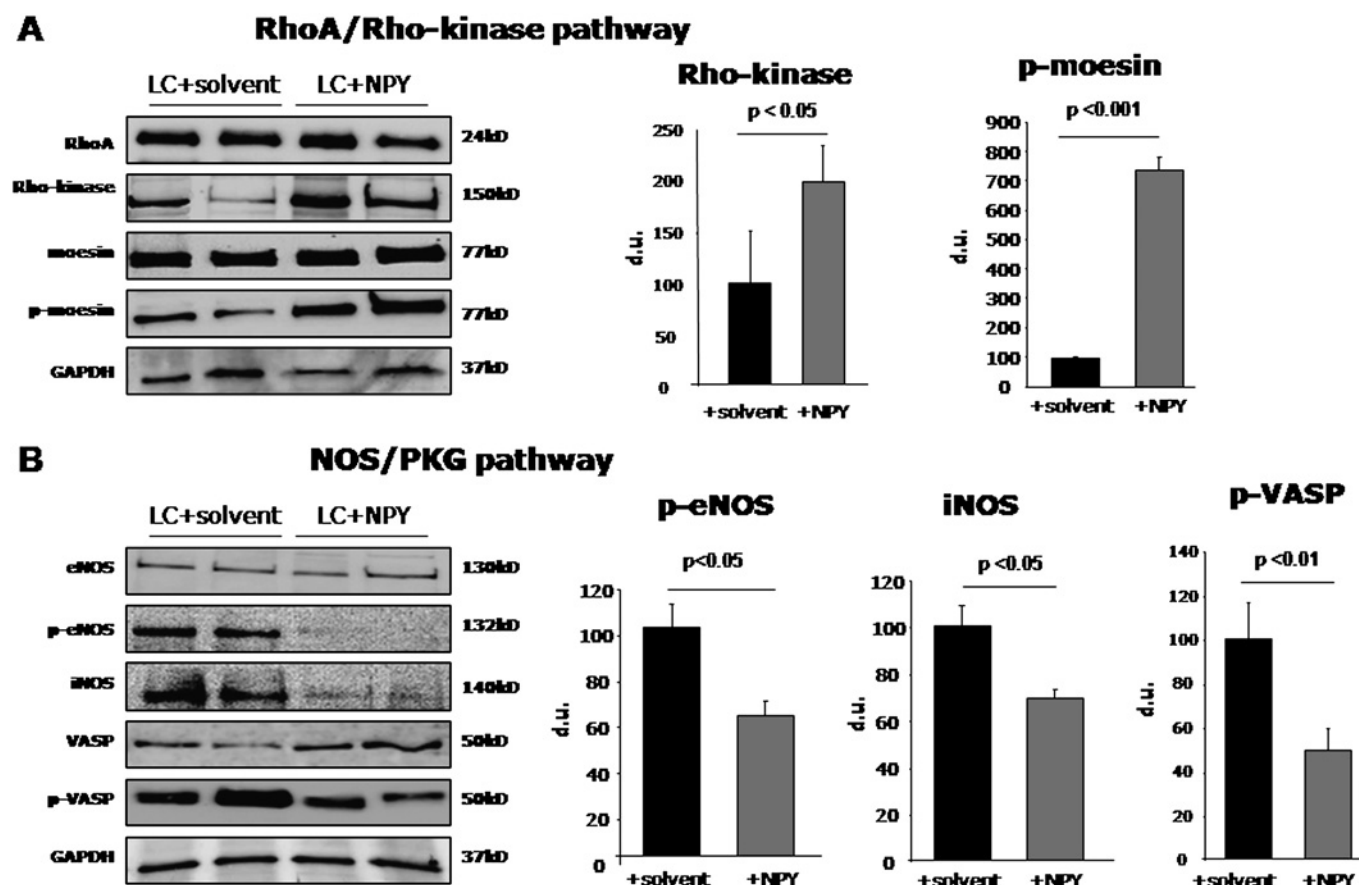
#### DISCUSSION

Vascular dysfunction in the splanchnic circulation during portal hypertension is characterised by vascular hyporeactivity to adrenergic stimulation and enhanced NO-mediated



**Figure 7** Vasomotor protein expression and activity in superior mesenteric artery in control rats without neuropeptide Y (NPY) (n=5) and after i.v. NPY administration (n=5). Mesenteric expressions of vasoconstrictive pathway proteins RhoA, Rho-kinase and moesin were determined by western blot analysis (A). Mesenteric expressions of vasodilating proteins eNOS, iNOS and VASP were analysed using western blot (B). Phosphorylation of moesin, eNOS, VASP, and endogenous control GAPDH were determined by western blot analysis using phospho- and site-specific antibodies. Shown are relative densitometric quantifications (means  $\pm$  SEM) of all experiments with values of controls set to 100 d.u. and representative western blots (minimum was n=5/group). d.u., density unit; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; LC, liver cirrhosis; PKG, protein kinase G; VASP, vasodilator-stimulated phosphoprotein.





**Figure 8** Vasomotoric protein expression and activity in superior mesenteric artery in cirrhotic rats without neuropeptide Y (NPY) (n=5) and after i.v. NPY administration (n=5). Mesenteric expressions of vasoconstrictive pathway proteins RhoA, Rho-kinase and moesin were determined by western blot analysis (A). Mesenteric expressions of vasodilating proteins eNOS, iNOS, VASP, and endogenous control GAPDH were analysed using western blot (B). Phosphorylation of moesin, eNOS and VASP were determined by western blot analysis using phospho- and site-specific antibodies. Shown are relative densitometric quantifications (means  $\pm$  SEM) of all experiments with values of controls set to 100 d.u. and representative western blots (minimum was n=5/group). d.u., density unit; eNOS, endothelial nitric oxide synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; iNOS, inducible nitric oxide synthase; LC, liver cirrhosis; VASP, vasodilator-stimulated phosphoprotein.

vasorelaxation leading to arterial vasodilation. Here, we report that NPY counteracts both of these key mechanisms. It decreases portal pressure and ameliorates hyperdynamic circulatory syndrome in cirrhotic rats with portal hypertension. First, NPY markedly augments vascular contractility in cirrhotic rats caused (among others) by enhancing Rho-kinase expression and activity, which counteracts vascular hyporeactivity. Second, NPY inhibits eNOS-, iNOS- and nitrgergic-induced NO production and its vasodilating effect on mesenteric arteries. Both effects are more pronounced in cirrhotic animals as compared to control rats. They explain the preferred effect of NPY in cirrhosis with portal hypertension.

The SNS mediates its vasoconstrictor action predominantly via the  $\alpha_1$ -adrenoceptor which represents the primary mechanism by which it controls total peripheral resistance.<sup>29</sup> In fact, in the mesenteric arterial bed in humans as well as in the animal species used in our experiments, vasoconstriction induced by neurally released norepinephrine is largely mediated by activation of postsynaptic  $\alpha_1$ -adrenoreceptors.<sup>29–30</sup> However, in humans and animals with cirrhosis and portal hypertension vasoconstrictors elicit less contraction in extrahepatic vessels at least partially due to a defective Rho-kinase pathway, which is responsible for maintaining vascular tone.<sup>10–12</sup> NPY sensitises the mesenteric vasculature to  $\alpha_1$ -adrenergic vasoconstriction and markedly improves contractility in pre-hepatic portal

hypertension.<sup>19</sup> Here, we confirm these results in an experimental model of liver cirrhosis known to present with a more marked hyperdynamic circulatory syndrome and more pronounced SNS activation than in pre-hepatic portal hypertension.<sup>31–32</sup> NPY decreases portal pressure by increases in splanchnic vascular resistance, as well as by attenuation of cardiac output (figures 1–3). Thus, it appears that NPY becomes increasingly important for optimising adrenergic vasoconstriction at particularly high adrenergic drive and plays a predominant role for vascular homeostasis.

We have reported previously that patients with cirrhosis present with elevated circulating plasma levels of NPY which correlate with portal pressure.<sup>33</sup> This finding indicates probably a compensatory mechanism to counterbalance arterial vasodilation through an enhanced NPY release and increased efficacy of endogenous catecholamines in the splanchnic circulation. Beside this beneficial effect, increased NPY levels might induce angiogenesis as recently shown in heart, brain and muscle,<sup>34–36</sup> and thus, may worsen portal hypertension due to increased portal inflow. Splanchnic angiogenesis has been shown to contribute largely to the development of the hyperdynamic circulation and to be mainly VEGF driven.<sup>37</sup> In this context, it is interesting to note that NPY has been suggested to act upstream of VEGF, and to represent a key factor for switching on the cascade of events leading to angiogenesis.<sup>35–38</sup> Most interestingly, NPY plasma

levels were found to be increased in cirrhotic patients already in compensated Child class A with no further increase in classes B or C<sup>33</sup> indicating a ceiling phenomenon of NPY release. Therefore, it is tempting to speculate that, in the early phase of portal hypertension increased NPY release may act as trigger for splanchnic angiogenesis. In later stages of the disease, the findings reported here support the hypothesis recently suggested that there is a downregulation or at least relative insufficiency in SNS activity in the splanchnic circulation.<sup>39</sup> This insufficiency of SNS and associated lack of vasoconstrictive action together with the reported increased activity of parasympathetic system might be partially responsible for the hyperdynamic circulation in advanced portal hypertension.<sup>40</sup> In vivo, exogenous NPY indeed reduced portal tributary blood flow only in cirrhotic animals and consequently ameliorated portal hypertension. In addition, NPY did not increase hepatoportal vascular resistance in cirrhotic animals arguing against a potential opposing intrahepatic effect. Moreover, NPY had no effect on MAP pointing towards a predominant splanchnic site of action of NPY.

We extend these in vivo observations by analysing the mechanisms in arterial mesenteric bed in vitro. These experiments show endothelium-independent beneficial effects of NPY in cirrhosis (figures 4 and 5) leading to an improvement in vascular contractility being not observed in healthy rats. In addition, the NPY-induced amplification of  $\alpha_1$ -adrenergic vasoconstriction was more pronounced in cirrhotic rats at high (but not low) levels of  $\alpha_1$ -adrenergic prestimulation. This points towards an improvement in the defective contractile signals of cirrhotic vessels.<sup>10–12</sup> Indeed, NPY increases Rho-kinase expression and activity in controls and cirrhotic rats (figures 7A, 8A). The decrease in Rho-kinase activity in extrahepatic cirrhotic vessels as shown earlier,<sup>10–12</sup> was substantially restored by NPY in cirrhotic rats. At least partially due to this effect mesenteric contractility in vitro was improved, leading to decreased portal inflow and portal pressure in vivo (figures 1A, 2A, 4B). This effect of NPY was unknown to date, and might explain why NPY promotes mesenteric contractility in portal hypertension.

Besides defective vasocontractile signals in extrahepatic vessels, endothelium derived NO plays an important role for arterial vasodilation in cirrhosis. In addition to its effects on Rho-kinase, NPY acts via the endothelium. It inhibited NOS induced vasorelaxation, and this effect was more pronounced in mesenteric arteries of cirrhotic rats as compared to control rats (figure 6). There are other studies on NPY, showing that it interacts with autonomic and sensory nervous vasodilatory effects beforehand. In detail, NPY has been shown to inhibit vasodilatory effects of ACh (parasympathetic), substance P (sensory peptide) as well as adenosine (sympathetic neurotransmitter) in various vascular beds.<sup>41–45</sup> Moreover, NPY has been shown to greatly reduce vasorelaxation in mesenteric arteries in response to PNS known to stimulate non-adrenergic and non-cholinergic (NANC) nerves.<sup>46</sup> Interestingly, the magnitude of blockade in eNOS- and PNS-induced vasorelaxation induced by NPY is increased in portal hypertensive conditions. Moreover, NPY abolishes the difference in eNOS- and nNOS-induced vasodilation between the study groups. Since, in portal hypertension mesenteric arteries exhibit eNOS, iNOS and nNOS upregulation<sup>4–7, 22</sup> it is tempting to speculate that NPY may interfere with this vascular NO overproduction. Indeed, Ishiwatari-Hayasaka *et al* recently reported a novel peptide binding region for NPY close to the N-terminal domain of HSP90 a key regulator of NOS activity.<sup>47</sup> In fact, among multiple biologically active peptides screened, NPY was found to exert the highest affinity and interaction with HSP90. In line with this, HSP90 has been shown to mediate in large parts the increased

eNOS-dependent and nitrergic vasorelaxation observed in mesenteric arteries in portal hypertension.<sup>49–50</sup> Our experiments endorse this hypothesis, since NPY administration decreased eNOS activation and NO-dependent vasodilation in mesenteric arteries, assessed as activity of the NO effector PKG (figures 7B, 8B). It is beyond the scope of this study to dissect the exact mechanism by which NPY increases Rho-kinase expression and activity. One might speculate that the potentiation of  $\alpha_1$ -adrenoceptor effect leads to intracellular pathways that (via G-protein coupled to RhoA) increase Rho-kinase expression and activity.

In summary, this study shows that acute intravenous administration of NPY reduces portal hypertension and ameliorates HCS in cirrhotic rats with severe portal hypertension. This effect is attributed to an improved arterial mesenteric contractility due to a novel dual cellular mechanism. NPY improves Rho-kinase activity in mesenteric arterial vascular wall and abrogates the NO overproduction in the mesenteric vasculature making NPY a superior vasoconstrictor counterbalancing arterial vasodilation in portal hypertension. Therefore, NPY appears as a new therapeutic option in humans with liver cirrhosis and portal hypertension.

**Acknowledgements** The authors gratefully acknowledge C. Hechtel, A. Sabo and G. Hack for the excellent technical support.

**Funding** This study was supported by the Regensburger Forschungsförderung in der Medizin (ReForM, to LM), the Deutsche Forschungsgemeinschaft (SFB TRR57 project 18, to JT) and BONFOR-Stiftung (to JT).

**Competing interests** None.

**Ethics approval** All experimental procedures in this study were conducted according to the German Physiological Society principles for the care and use of laboratory animals (Granted permission number 621–2531.1–23/00, government of Bavaria).

**Provenance and peer review** Not commissioned; externally peer reviewed.

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## Editor's quiz: GI snapshot

### ANSWER

From question on page 1067

Figures 1 and 2 demonstrate intrahepatic histoacryl glue and lipoidal deposition in all branches of the intrahepatic portal vein but with patent extrahepatic portal vasculature. Splenomegaly and splenic varices are evident. The portal pressure study results are consistent with segmental left-sided portal hypertension. The histoacryl glue and lipoidal were deposited in the intrahepatic portal veins following drainage along a pressure gradient from the short gastric varices via the left gastric system into the portal vein.

Gastric varices resulting from segmental splenic hypertension are a rare and challenging cause of upper gastrointestinal bleeding especially in patients with a pre-existing diagnosis of underlying liver disease.<sup>1</sup> A splenic vein thrombosis secondary to pancreatic disease is the most common aetiology, but multiple rare causes have been reported.<sup>2</sup> Pressure studies are diagnostic with elevated pressures limited to the splenic and short gastric

veins. Conventional treatment for variceal haemorrhage is often unsuccessful and splenectomy is usually required.<sup>3</sup> Patients without a malignant cause usually have an excellent prognosis due to the absence of significant underlying liver disease.<sup>4</sup> Our patient ultimately underwent a splenectomy and remained well on follow-up.

*Gut* 2011;**60**:1132. doi:10.1136/gut.2010.226902

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# Amelioration of portal hypertension and the hyperdynamic circulatory syndrome in cirrhotic rats by neuropeptide Y via pronounced splanchnic vasoaction

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*Gut* 2011 60: 1122-1132 originally published online February 12, 2011  
doi: 10.1136/gut.2010.226407

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