Effect of short- and long-term portal hypertension on adrenergic, nitrergic and sensory functioning in rat mesenteric artery

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ABSTRACT

In the present study, we analysed possible alterations in adrenergic, nitrergic and sensory functioning in mesenteric arteries from rats at 1 and 21 months after partial portal vein ligation, and the mechanisms involved in these alterations, if any. For this purpose, we analysed the vasoconstrictor response to EFS (electrical field stimulation) and the effect of the α-antagonist phentolamine, the NOS (nitric oxide synthase) inhibitor L-NAME (N(G)-nitro-L-arginine methyl ester) and the CGRP (calcitonin gene-related peptide) receptor antagonist CGRP-(8–37) in mesenteric segments from ST (short-term; 1 month) and LT (long-term; 21 months) SO (sham-operated) and pre-hepatic PH (portal hypertensive) rats. The vasoconstrictor responses to NA (noradrenaline), the NO donor DEA-NO (diethylamine NONOate) and CGRP were analysed. NA, NO and CGRP releases were measured. Phospho-nNOS (neuronal NOS) expression was studied. The vasoconstrictor response to EFS was decreased in STPH animals. Phentolamine decreased this vasoconstrictor response more strongly in SO animals. Both L-NAME and CGRP-(8–37) increased vasoconstrictor response to EFS more strongly in PH than SO segments. PH did not modify vasomotor responses to NA, DEA-NO or CGRP, but it decreased NA release while increasing those of NO and CGRP. Phospho-nNOS expression was increased by PH. In LTPH, no differences were observed in vasoconstrictor response to EFS, vasomotor responses or neurotransmitter release when compared with age-matched SO animals. In conclusion, the mesenteric innervation may participate in the development of the characteristic hyperdynamic circulation observed in STPH through the joint action of decreased adrenergic influence, and increased nitrergic and sensory innervations influences. The participation of each innervation normalizes under conditions of LTPH.

INTRODUCTION

PH (portal hypertension) is a clinical syndrome defined, among other factors, by a pathologically altered splanchnic and systemic blood flow brought about by an increased vasodilation associated with endothelial factor alterations [1,2]. This increased vasodilation is also present when PH is maintained for a long time.
In fact, we have previously demonstrated increased endothelial prostacyclin participation in aorta vasodilation observed in LT (long-term) PH [5]. Nevertheless, vascular tone is the result of an equilibrium among several mechanisms, and the adrenergic, cholinergic, nitrergic, peptidergic and/or sensory innervations play important functional roles that depend on the vascular bed being analysed. The rich sympathetic [7], sensory [8] and nitrergic innervations [9,10] of rat mesenteric arteries modulate vascular tone by releasing the vasconstrictor neurotransmitter NA (noradrenaline), and the vasodilator neurotransmitters CGRP (calcitonin gene-related peptide) and NO under electrical stimulation.

We have reported that different physiopathological situations, such as diabetes [11,12], hypertension [13,14], cirrhosis [15] or aging [16,17], can modify the participation of the different innervations in the vasomotor response to EFS (electrical field stimulation). In PH, and with regard to adrenergic innervation, both an increase and a decrease in vasoconstrictor response to NA have been reported [4,18–21], as well as a down-regulation of genes related to the adrenergic system and atrophy of mesenteric sympathetic innervation [22,23]. In reference to nitrergic innervation, increased involvement of neuronal NO in EFS-induced vasodilation [24] and in nNOS [neuronal NOS (nitric oxide synthase)] expression [25] has been described. Additionally, an increased CGRP participation in periarterial nerve stimulation has been suggested [24]. In cirrhotic rats, we have reported a pivotal role by CGRP in the decreased vasoconstrictor response induced by EFS, mediated by an increase in CGRP release and vasodilator response [15]. In summary, few studies have analysed the function of each innervation in PH or their integrated effect on vascular tone, whereas the evolution of their participation in a situation of LT maintenance of PH is also a question of some interest. Since PH can last for several years in humans, the aim of this study is to analyse the impact of both STPH (short-term PH) and LTPH on periarterial nerve stimulation function.

The present study was designed to analyse possible alterations in adrenergic, nitrergic and sensory function in mesenteric arteries from rats at 1 and 21 months after partial portal vein ligation, and the mechanisms involved in any alterations.

**MATERIALS AND METHODS**

**Animals**

The study was conducted in male Wistar rats (250 g; Harlan Ibérica SL), according to the European Union guidelines for ethical care of experimental animals and U.S. National Institutes of Health guidelines. Animals were fed rat chow and water ad libitum. They were housed in groups of 3–4 animals in a light/dark-controlled room, with an average temperature (22 ± 2 °C) and humidity (65–70%). Animals were randomly divided into two groups: SO (sham-operated) rats and rats with pre-hepatic PH produced by triple partial portal vein ligation. These animals were maintained for 1 month (STPH) or 21 months (LTPH).

**Surgical techniques**

Rats were anaesthetized by intraperitoneal injection of ketamine (100 mg/kg of body weight) and Xylacine (12 mg/kg of body weight). The surgical procedure to produce PH by triple partial ligation of the portal vein has been described previously [26]. In brief, a midline abdominal incision was made, the portal vein isolated and three ligatures, fixed on a sylactic guide, were performed in its superior, middle and inferior portions. The stenoses were calibrated by a simultaneous ligation (4-0 silk) around the portal vein and a 20-gauge blunt-tipped needle. The midline incision was closed in two layers with an absorbable suture (polyglycolic acid) and 3-0 silk. Analgesia was maintained during 24 h with Buprenorphine (0.05 mg/kg, 8 h subcutaneous). In SO rats, the procedure was the same except for ligation of the portal vein.

Systolic blood pressure was measured using the tail-cuff method before killing. Animals were anaesthetized by intraperitoneal injection of ketamine (100 mg/kg of body weight) and xylacine (12 mg/kg of body weight), a midline abdominal incision was made and PP (portal pressure) was registered and mesenteric venous vasculopathy and collateral circulation were studied. At the end of the experiments, each animal was killed by exanguination through the vena cava at 1 month (ST) or at 21 months (LT). The superior mesenteric artery was carefully dissected out, cleaned of connective tissue, cut into 4-mm-long segments and placed in KHS (Krebs–Henseleit solution; 115 mmol/l NaCl, 2.5 mmol/l CaCl₂, 4.6 mmol/l KCl, 1.2 mmol/l KH₂PO₄, 1.2 mmol/l MgSO₄, 25 mmol/l NaHCO₃, 11.1 mmol/l glucose and 0.03 mmol/l Na₂EDTA) at 4 °C.

**PP measurement**

Splenic pulp pressure, an effective indirect measurement of PP, was measured as described previously [5,15] by inserting a 23-gauge fluid-filled needle into the spleen parenchyma. The needle was joined to a PE-50 tube, and then connected to a pressure recorder (PowerLab 200 ML 201) and a transducer (Sensonor SN-844) with a Chart V4.0 computer program (AD Instruments); the recorder was re-calibrated before each experiment.

**Mesenteric venous vasculopathy and portosystemic collateral circulation study methods**

The existence of high PP was confirmed by the development of mesenteric venous vasculopathy and...
collateral portal-systemic circulation and was observed only in PH rats. First, a midline abdominal incision with a large bilateral subcostal extension was performed. Mesenteric venous vasculopathy, a characteristic feature of splanchnic venous congestion, manifests as distension and tortuosity of the superior mesenteric vein branches [27]. Portosystemic collateral circulation was studied by macroscopic examination of the areas in which the collateral venous circulation had developed, and the splenorenal, gastroesophageal, colorectal and hepatic hilum were carefully studied for the presence of increased collateral veins [28].

Vascular reactivity
The method used for isometric tension recording has been described in full elsewhere [10, 29]. Briefly, two parallel stainless steel pins were introduced through the lumen of the vascular segment: one was fixed to the bath wall, and the other connected to a force transducer (Grass FT03C); this was connected in turn to a model 7D Grass polygraph. For EFS experiments, segments were mounted between two platinum electrodes 0.5 cm apart and connected to a stimulator (model S44; Grass) modified to supply the appropriate current strength. Segments were suspended in an organ bath containing 5 ml of KHS at 37 °C continuously bubbled with a 95% O2/5% CO2 mixture (pH 7.4). Experiments were performed in endothelium-denuded segments to eliminate the main source of vasoactive substances, including endothelial NO. This avoided possible actions by different drugs on endothelial cells that could have led to result misinterpretation. Endothelium was removed by gently rubbing the luminal surface of the segments with a thin wooden stick. The segments were subjected to a tension of 0.5 g which was readjusted every 15 min during a 90-min equilibration period before drug administration. After this, the vessels were exposed to 75 mmol/l KCl to check their functional integrity. Endothelium removal did not alter the contractions elicited by 75 mmol/l KCl. After a washout period, the absence of vascular endothelium was proven by the inability of 10 μmol/l ACh (acetylcholine) to relax segments pre-contracted with NA (995 ± 1.29 mg).

Frequency-response curves to EFS (1, 2, 4, 8 and 16 Hz) were performed. The parameters used for EFS were 200 mA, 0.3 ms, 1–16 Hz, for 30 s with an interval of 1 min between each stimulus, the time required to recover basal tone. A washout period of at least 1 h was necessary to avoid desensitization between consecutive curves. Three successive frequency–response curves separated by 1 h intervals produced similar contractile responses. To evaluate the neural origin of the EFS-induced contractile response, the nerve impulse propagation blocker TTX (tetrodotoxin; 0.1 μmol/l) [10] was added to the bath 30 min prior to performing the second frequency–response curve.

To determine the participation of adrenergic innervation in the EFS-induced response in segments from all experimental groups, 1 μmol/l phenotolamine [10], an α-adrenoceptor antagonist, was added to the bath 30 min before performing the frequency–response curve. Additionally, the vasoconstrictor response of exogenous NA (1 nmol/l to 10 μmol/l) was tested in segments from all experimental groups.

To analyse the participation of nitricergic innervation in the EFS-induced response in segments from all rat groups, 0.1 mmol/l L-NAME (Nω-nitro-L-arginine methyl ester) [10], a non-specific inhibitor of NOS, was added to the bath 30 min before performing the second frequency–response curve. To rule out the inducible origin of NO, a similar protocol was performed with the specific iNOS (inducible NOS) inhibitor 1400W (1 μmol/l) [30]. The vasodilator response to the NO donor, DEA-NO (diethylamine NONOate; 0.1 nmol/l to 0.1 mmol/l) was determined in NA-pre-contracted arteries from all experimental groups.

To study the possible participation of sensitive innervation in the EFS-induced response in segments from all experimental groups, 0.5 μmol/l CGRP-(8–37) [31], a CGRP receptor antagonist, was added to the bath 30 min before performing the second frequency–response curve. The vasodilator response to exogenous CGRP (0.1 nmol/l to 0.1 μmol/l) was analysed in NA-pre-contracted segments from all animal groups.

NA and CGRP release
Endothelium-denuded mesenteric segments from STSO, STPH, LTSO and LTPH rats were pre-incubated for 30 min in 5 ml of KHS at 37 °C and continuously bubbled with a 95% O2/5% CO2 mixture (stabilization period). This was followed by two washout periods of 10 min in a bath of 0.4 ml of KHS, after which arteries were subjected to cumulative EFS periods of 30 s at 1, 2, 4, 8 and 16 Hz at 1 min intervals, as described above. The release of NA and CGRP was measured by using an NA research EIA (enzyme immunoassay) kit (Labor Diagnostica Nord) and a rat CGRP enzyme immunoassay kit (Spibio) respectively. The different assays were performed following manufacturers’ instructions. Results are expressed as ng of NA/ml per mg of tissue and pg of CGRP/ml per mg of tissue.

NO release
After an equilibration period of 60 min in Hepes buffer (119 mmol/l NaCl, 20 mmol/l Hepes, 1.2 mmol/l CaCl2, 4.6 mmol/l KCl, 1 mmol/l MgSO4, 0.4 mmol/l KH2PO4, 5 mmol/l NaHCO3, 5.5 mmol/l glucose and 0.15 mmol/l Na3HPO4, pH 7.4) at 37 °C, endothelium-denuded mesenteric segments from each experimental group were incubated with the fluorescent probe 4,5-diaminofluorescein (DAF-2; 2 μmol/l) for 45 min. Then, the medium was collected to measure basal NO release.
Afterwards, cumulative EFS periods of 30 s at 1, 2, 4, 8 and 16 Hz were applied at 1 min intervals. The fluorescence of the medium was measured at room temperature (22–25°C) using a spectrofluorimeter (LS50 with FL WINLAB Software; PerkinElmer) with λ_{ex} = 492 nm and λ_{em} = 515 nm.

The EFS-induced NO release was calculated by subtracting basal NO release from that evoked by EFS. In addition, blank samples were collected in the same way from segment-free medium in order to subtract background emission. Some assays were performed in the presence of 0.1 mmol/l L-NAME, 1 μmol/l 1400W or 0.1 μmol/l TTX to ensure the specificity of the method. The amount of released NO was expressed as arbitrary units/mg of tissue.

Western blot analysis
Endothelium-denuded mesenteric segments from all groups of rats were homogenized in a boiling buffer composed of 1 mM sodium vanadate, 1% SDS, and 0.01 M Tris/HCl, pH 7.4. Homogenates containing 40 μg of protein were electrophoretically separated by SDS/PAGE (7.5% gel), and then transferred on to PVDF membranes (Immun-Blot; Bio-Rad Laboratories) overnight at 4°C and 230 mA using a Bio-Rad Mini Protean III system (Bio-Rad Laboratories) containing 25 mM Tris, 190 mM glycine, 20% methanol and 0.05% SDS. The membrane was blocked for 1 h at room temperature in Tris-buffered saline (100 mM Tris/HCl, pH 7.4, 0.9%, NaCl and 0.1% SDS) with 5% BSA before being incubated overnight at 4°C with a rabbit monoclonal antibody against nNOS (1:1000 dilution; Abcam) or a rabbit polyclonal antibody against phospho-nNOS (1:1000 dilution; Abcam). After washing, the membranes were incubated with anti-rabbit HRP (horseradish peroxidase)-conjugated IgG antibody (GE Healthcare). The membrane was thoroughly washed and the immunocomplexes were then detected using an enhanced HRP/luminol chemiluminescence system (ECL Plus; GE Healthcare) and subjected to autoradiography (Hyperfilm ECL, GE Healthcare). Signals on the immunoblot were quantified using a computer program (NIH Image version 1.56; National Institute of Health, Bethesda, MD, U.S.A.). The same computer program (NIH Image version 1.56; National Institute of Health, Bethesda, MD, U.S.A.) was used to determine α-actin expression, and the content of the latter was used to correct protein expression in each sample by means of a monoclonal anti-α-actin antibody (1:2000 dilution; Sigma). Rat brain homogenate was used as positive control.

Drugs used
L-NA hydrochloride, ACh chloride, DEA-NONOate diammonium salt, CGRP-(8–37), rat CGRP, TTX, L-NAME hydrochloride, phenolamine, 1400W, tempol and DAF-2 were from Sigma. Stock solutions (10 mmol/l) of drugs were made in distilled water, except for NA which was dissolved in a 0.9% NaCl/0.01% ascorbic acid. These solutions were kept at −20°C and appropriate dilutions were made in KHS on the day of the experiment.

Data analysis
The responses elicited by EFS or NA are expressed as a percentage of the initial contraction elicited by 75 mmol/l KCl for comparison between SO and PH rats. The relaxation induced by DEA-NO or CGRP is expressed as a percentage of the initial contraction elicited by NA. Results are given as means ± S.E.M. Statistical analysis was done by comparing the curve obtained in the presence of the different substances with the previous or control curve by means of repeated measure (two-way ANOVA). The analysis of individual points of each curve was analysed using Bonferroni post-hoc test. For the experiments on NO, CGRP and NA release, the statistical analysis was done using one-way ANOVA, followed by Newman–Keuls post-hoc test. P < 0.05 was considered significant.

RESULTS

Animal evolution
STPH and LTPH did not modify SBP (systolic blood pressure), although it did raise PP (Table 1). Body, spleen and liver weights were similar in both ST and LT SO and PH animals (Table 1). All rats in the STPH and LTPH groups presented portosystemic collateral circulation (pararectal, paraoesophageal and splenorenal collateral vessels).

Vascular reactivity
The response induced by 75 mmol/l KCl was similar in segments from all rat groups (STSO, 1184 ± 79.77 mg; STPH, 1010 ± 92.67 mg; LTSo: 1447 ± 96.34 mg; LTPH: 1322 ± 108.1 mg; P > 0.05; n = 10 animals each group). The contractions elicited by EFS were lower in mesenteric segments from STPH rats than those from STSO rats (P < 0.05; Figure 1A). EFS-induced contractions were similar in segments from LTSo and LTPH animals (P > 0.05; Figure 1B). Pre-incubation with the nerve impulse propagation blocker TTX (0.1 μmol/l) practically abolished EFS-induced contractions in segments from all experimental groups (Figure 2).

The contraction elicited by EFS was significantly reduced by the α-adrenoceptor antagonist, phentolamine (1 μmol/l), in segments from all rat groups (Figure 3). This decrease was greater in segments from STSO than from STPH rats. (Figures 3A and 3B, and Table 2). Phentolamine decreased EFS-induced vasoconstriction to a similar extent in segments from both LT groups (Figures 3C and 3D, and Table 2).
Table 1  Modification of body weight, SBP, spleen weight and liver weight in STSO, STPH, LTSO and LTPH rats
Results are expressed as means ± S.E.M.; n = 10 animals in each group. *P < 0.05 compared with age-matched STSO.

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (g)</th>
<th>SBP (mmHg)</th>
<th>PP (mmHg)</th>
<th>Spleen weight/body weight (%)</th>
<th>Liver weight/body weight (%)</th>
</tr>
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<tbody>
<tr>
<td>STSO</td>
<td>337.4 ± 6.6</td>
<td>123.4 ± 1.8</td>
<td>5.45 ± 0.77</td>
<td>0.24 ± 0.07</td>
<td>2.4 ± 0.1</td>
</tr>
<tr>
<td>STPH</td>
<td>330.2 ± 6.1</td>
<td>119.4 ± 1.2</td>
<td>9.90 ± 0.64*</td>
<td>0.30 ± 0.01</td>
<td>2.5 ± 0.06</td>
</tr>
<tr>
<td>LTSO</td>
<td>617.3 ± 83.5</td>
<td>137.1 ± 2.2</td>
<td>9.01 ± 1.14</td>
<td>0.22 ± 0.06</td>
<td>2.56 ± 0.31</td>
</tr>
<tr>
<td>LTPH</td>
<td>629.9 ± 86.4</td>
<td>120.5 ± 7.6</td>
<td>12.68 ± 1.27*</td>
<td>0.18 ± 0.07</td>
<td>2.53 ± 0.25</td>
</tr>
</tbody>
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Figure 1  Isometric tension recording of the frequency–dependent contractions in denuded mesenteric artery segments from STSO and STPH rats (A), and LTSO and LTPH rats (B)
Results (means ± S.E.M.) are expressed as a percentage of tone induced by 75 mmol/l KCl; n = 10 animals in each group. *P < 0.05 compared with control animals for each frequency (Bonferroni test).

Figure 2  Effect of 0.1 μmol/L TTX on the frequency–response curves performed in endothelium-denuded mesenteric artery segments from STSO, STPH, LTSO and LTPH rats
(A and C) Results from SO animals; (B and D) results from PH rats. Results (means ± S.E.M.) are expressed as a percentage of a previous tone with 75 mmol/l KCl; n = 10 animals in each group. *P < 0.05 compared with conditions without specific inhibitor at each frequency (Bonferroni test).
### Table 2  Respective EFS inhibition/potentiation after pre-incubation with 1 μmol/l phentolamine or 0.1 mmol/l L-NAME

Results are expressed as means ± S.E.M. of the percentage inhibition/potentiation of EFS-induced contraction after pre-incubation with 1 μmol/l phentolamine or 0.1 mmol/l L-NAME in STSO, STPH, LTSO and LTPH animals; n = 10 animals in each group. Calculations are performed taking the control EFS-induced contraction as 100% of the contractile response. *P < 0.05 compared with age-matched SO.

<table>
<thead>
<tr>
<th>Group</th>
<th>EFS inhibition/potentiation (%)</th>
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<tbody>
<tr>
<td></td>
<td>1 Hz</td>
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<tr>
<td>STSO + phentolamine</td>
<td>100 ± 0.1</td>
</tr>
<tr>
<td>STPH + phentolamine</td>
<td>100 ± 0.2</td>
</tr>
<tr>
<td>LTSO + phentolamine</td>
<td>100 ± 0.1</td>
</tr>
<tr>
<td>LTPH + phentolamine</td>
<td>100 ± 0.2</td>
</tr>
<tr>
<td>STSO + L-NAME</td>
<td>38.5 ± 2.9</td>
</tr>
<tr>
<td>STPH + L-NAME</td>
<td>105.4 ± 2.8*</td>
</tr>
<tr>
<td>LTSO + L-NAME</td>
<td>40.3 ± 3.9</td>
</tr>
<tr>
<td>LTPH + L-NAME</td>
<td>39.1 ± 2.1</td>
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**Figure 3** Effect of 1 μmol/l phentolamine on the frequency–response curves performed in endothelium-denuded mesenteric artery segments from STSO, STPH, LTSO and LTPH rats

(A and C) Results from SO animals; (B and D) results from PH rats. Results (means ± S.E.M.) are expressed as a percentage of a previous tone with 75 mmol/l KCl; n = 10 animals in each group. *P < 0.05 compared with conditions without specific inhibitor at each frequency (Bonferroni test).

The contraction induced by EFS was significantly increased by pre-incubation with the NOS inhibitor L-NAME (0.1 mmol/l) in segments from all experimental groups (Figure 4). This increase was greater in segments from STPH compared with STSO (Figures 4A and 4B, and Table 2). L-NAME increased EFS-induced vasoconstriction to a similar extent in segments from LTSO and LTPH animals (Figures 4C and 4D). Pre-incubation with specific iNOS inhibitor 1400W (1 μmol/l) did not modify EFS-induced response in any experimental group (Figure 4).

The specific CGRP receptor antagonist CGRP-(8–37) (0.5 μmol/l) did not modify the contractile response induced by EFS in segments from STSO rats (Figure 5A), but it increased the response in segments from STPH rats (Figure 5B). Pre-incubation with CGRP-(8–37) did not modify the contractile response induced by EFS in LTSO or LTPH animals (Figures 5C and 5D).

PH did not modify vasoconstrictor responses to exogenous NA (1 nmol/l to 10 μmol/l) or vasodilator responses to NO donor DEA-NO (0.1 nmol/l to 0.1 μmol/l) and exogenous CGRP (0.1...
Figure 4 Effect of 0.1 mmol/l L-NAME or 1 μmol/l 1400W on the frequency–response curves performed in endothelium-denuded mesenteric artery segments from STSO, STPH, LTSO and LTPH rats
(A and C) Results from SO animals; (B and D) results from PH rats. Results (means ± S.E.M.) are expressed as a percentage of a previous tone with 75 mmol/l KCl. n = 10 animals in each group. *P < 0.05 compared with conditions without specific inhibitor at each frequency (Bonferroni test).

nmol/l to 0.1 μmol/l) in either ST or LT animals (Figure 6).

NA release
Basal NA release was lower in mesenteric segments from STPH than from STSO rats (Figure 7A). EFS increased NA release in STSO and STPH arteries, but more strongly in segments from SO rats (Figure 7A).

Both basal and EFS-induced NA releases were similar in segments from LTSO and LTPH animals (Figure 7A).

CGRP release
In ST animals, basal CGRP release was higher in segments from PH rats than in the SO animals (Figure 7B). EFS increased CGRP levels in both groups of rats, but more so in segments from PH rats (Figure 7B).

Expression of nNOS and phospho-nNOS
A 155 kDa band was detected in nNOS analysis. nNOS expression was similar in homogenates from PH rats and SO rats at 1 month (Figure 7C). Phospho-nNOS expression was detected as a 160 kDa band, and was higher in segments from STPH rats compared with SO rats (Figure 7C).

NO release
Basal NO release was higher in mesenteric segments from STPH than from STSO rats. EFS increased NO release in both groups of rats, but more notably in segments from PH rats (Figure 7D). Both basal and EFS-induced NO releases were similar in LTSO and LTPH rats (Figure 7D). Pre-incubation with 0.1 mmol/l L-NAME or with 0.1 μmol/l TTX abolished NO release by EFS in arteries from all experimental groups, whereas 1400W (1 μmol/l) did not modify NO release (Figure 8).

DISCUSSION
Splanchnic vasodilation is the pathophysiological hallmark of the development of the hyperdynamic circulatory syndrome [18,32,33] in PH. This splanchnic vasodilation has been described to be mainly due to alterations in endothelial factors, the participation of which changes over time [3–5]. Despite the important role of perivascular innervation few studies have been performed on the possibility that alterations occur in the adrenergic, nitrergic and sensory innervations, and their integrated effects on mesenteric vascular tone. Thus, the objective of the present study was to determine whether STPH and LTPH could modify the potential functional influence of the different types of mesenteric innervation.

The vasoconstrictor response to KCl, an indicator of smooth muscle functional integrity, was not modified by the development of STPH or LTPH, in contrast with previous reports. This apparent contradiction may reflect the different experimental models used [34,35].
Figure 5  Effect of 0.5 μmol/l CGRP-(8–37) on the frequency–response curves performed in endothelium-denuded mesenteric artery segments from STSO, STPH, LTSO and LTPH rats
(A and C) Results from SO animals; (B and D) results from PH rats. Results (means ± S.E.M.) are expressed as a percentage of a previous tone with 75 mmol/l KCl. n = 10 animals each group.

Our results show that EFS induced a contractile responses in endothelium-denuded mesenteric segments from all experimental groups. This vasoconstrictor response elicited by EFS was practically abolished by the blocker for nerve impulse propagation TTX, demonstrating that the response is due to the release of neurotransmitters from the nerve endings.

Short-term PH

The operated rats had portal hyperpressure and developed systemic collateral vessels, thus confirming the validity of triple partial portal vein ligation as an experimental model to establish PH [27,28].

In STPH rats, the level of vasoconstrictor response to EFS was lower than in SO animals. This diminished vasoconstrictor response suggests neural modifications associated with PH. This effect could be associated with changes in the release of and/or response to the different neurotransmitters.

The participation of adrenergic innervation in the EFS-induced vasoconstriction was analysed by incubation with the α-adrenoceptor blocker phentolamine. The vasoconstrictor response was significantly reduced by phentolamine in STSO and STPH rats, confirming that this response is at least mediated by NA release from adrenergic nerve terminals and the subsequent activation of α-adrenoceptors. The decrease was lower in segments from PH animals, indicating a lower participation of adrenergic innervation in these animals. Our next objective was to analyse whether the decreased adrenergic participation in STPH was associated with a decrease in NA release and/or vasoconstrictor response to NA. In our experimental conditions, concentration-response curves to exogenous NA were similar in segments from both groups of rats, contrasting with previous studies reporting both an increase and a decrease of vasoconstrictor response to NA [4,18–21]; meanwhile, both basal and EFS-induced NA releases were diminished in mesenteric segments from PH rats, as also reported with reference to circulating NA [18,19]. Therefore these results explain the decrease of adrenergic function that results in the diminished vasoconstrictor response to EFS observed in STPH.

Although the diminished adrenergic function could, by itself, explain the decreased EFS vasoconstriction, the participation of other neural components cannot be ruled out. The NO released from nitrergic innervation in EFS has been demonstrated to have a vasodilator role contributing to the vasomotor response to EFS [10–14]. In vascular reactivity experiments, pre-incubation with 7-NI (7-nitroindazole) decreases vasoconstrictor response to NA, making the analysis of EFS-induced contractions very complex [36,37]. For that reason, we used the non-selective NOS inhibitor L-NAME in vascular reactivity experiments. EFS-induced vasoconstriction was increased in both experimental groups by pre-incubation with L-NAME. This increase was higher in mesenteric segments from PH than in SO rats, suggesting that the participation of nitrergic innervation is increased in PH; this could be the result of increased NO release and/or altered smooth muscle cell sensitivity to NO. The fact that the vasodilator response to the NO analogue
DEA-NO was similar in both experimental groups rules out differences in smooth muscle sensitivity to NO. However, we showed directly that both basal and EFS-induced NO release were increased in PH compared with SO rats, in agreement with Jurzic et al. [25]. This augmented NO release is abolished by TTX, thus confirming the neural origin of the NO, and indicating that neuronal NO also participates in the effect of PH. Production of NO in neural tissue can have two sources: nNOS and iNOS [36–38]. However, in the present study, the specific iNOS inhibitor 1400W did not change either the vasoconstrictor response to EFS or the NO release in segments from the two experimental groups, ruling out the participation of this inducible isoform and suggesting that NO release was, indeed, dependent on nNOS expression and/or activity. We found that nNOS protein expression was not modified by PH, contrasting with previous reports [25,39], whereas the active form phospho-nNOS was increased in PH, suggesting that PH had increased nNOS activity.

Once the participation of both adrenergic and nitrergic innervation in the diminished response to EFS in segments from PH rats had been demonstrated, we studied the possible role of sensory innervation in this vasomotor response. We have previously reported that in this strain sensory innervation does not participate in vasomotor response induced by EFS in control animals, but that the participation of this innervation is increased in several pathological conditions such as hypertension, cirrhosis and diabetes [12,15,40,41]. In the present study, we observed that pre-incubation with the CGRP antagonist CGRP-(8–37) did not modify the vasoconstrictor response to EFS in mesenteric rings from SO rats, confirming CGRP non-participation in vasconstriction in healthy Wistar rats [10,36,42]. However, CGRP-(8–37) increased the vasoconstrictor response to EFS in segments from PH rats. These results indicate that sensory innervation participates in the vasomotor response to EFS in PH rats, indicating a role for sensory innervation in the decreased vasoconstrictor response to EFS in STPH. Thus our next objective was to analyse whether this participation was associated with an increase in the CGRP release and/or vasodilatory response. The vasodilator response to exogenous CGRP was similar in segments from both experimental groups, ruling out differences in sensitivity to CGRP due to PH, meanwhile both basal and EFS-induced CGRP releases were higher in mesenteric segments from PH rats, indicating that sensory innervation also contributed to the diminished vasoconstrictor response to EFS in PH through an increase in CGRP release.

Altogether, these results confirm that in STPH the EFS-induced vasoconstrictor response in mesenteric arteries is decreased as a result of decreased adrenergic innervation function and increased sensory and nitrergic innervation function.
Figure 7  Neurotransmitter release and Western blot analysis

(A) Effect of STPH and LTPH on basal and EFS-induced NA release in endothelium-denuded mesenteric segments; n = 6 animals each group. Results are expressed as ng of NA/ml per mg of tissue. ∗ P < 0.05 compared with basal; #P < 0.05 compared with SO. (B) Effect of STPH on basal and EFS-induced CGRP release in mesenteric segments. Results are expressed as pg of CGRP/ml per mg of tissue; n = 6 animals. ∗ P < 0.05 compared with basal; #P < 0.05 compared with SO. (C) Western blot for phospho-nNOS and nNOS expression in endothelium-denuded mesenteric artery segments from STSO and STPH rats. The Figure is representative of preparations from six rats in each group. Lower panel shows relation between densitometric analysis for phospho-nNOS compared with nNOS expression. (D) Effect of STPH and LTPH on basal and EFS-induced NO release in endothelium-denuded mesenteric segments; n = 6 animals in each group. Results are expressed as arbitrary units (A.U.)/mg of tissue. ∗ P < 0.05 compared with basal; #P < 0.05 compared with STSO.

Figure 8  Effect of 1 μmol/l 1400W, 0.1 mmol/l L-NAME or 0.1 μmol/l TTX on basal and EFS-induced NO release in endothelium-denuded mesenteric segments from (A) STSO, (B) STPH, (C) LTSO and (D) LTPH rats

Results are expressed as arbitrary units (A.U.)/mg of tissue; n = 6 animals in each group. ∗ P < 0.05 compared with basal; #P < 0.05 compared with conditions without specific inhibitor.
Long-term PH
The operated rats presented portal hyperpressure and had systemic collateral vessels at 21 months after portal vein ligation. The persistence in these changes related to PH indicates that triple partial portal vein ligation in the rat is an appropriate experimental model that maintains its validity even during very late stages, making it possible to establish a correlation with human PH, as previously suggested [5]. In later stages, an increase in splanchnic blood flow leads to the hyperdynamic circulation state, which in turn contributes to the maintenance and aggravation of many of the complications of PH [43]. In this sense, we have reported previously that maintenance of PH for 21 months also maintains the changes in endothelial function that result from increased prostacyclin release, and contributes to maintaining the increased vasodilator response to ACh [5]. Consequently, our next aim was to determine whether the different innervations also play a role in the hyperdynamic circulation present in LTPH.

The vasoconstrictor response to EFS is stronger in LTSO rats than in STSO animals as a consequence of aging, as we have previously reported [16]. In contrast with the decrease observed in STPH rats, the vasoconstrictor response to EFS is not modified in LTPH rats compared with their age-matched SO animals. This result indicates a reversion of the decreased vasoconstrictor effect on innervation in STPH, and contrasts with its effect on endothelial function [5]. However, a rearrangement of the participation of each kind of innervation cannot be ruled out.

In both experimental groups, phenolamine reduced the vasoconstrictor response to EFS to a similar extent, and both the vasoconstrictor response to NA and NA release were similar in both SO and PH rats, indicating a restored function of the adrenergic innervation at 21 months. EFS-induced vasoconstriction was similarly increased by 1-NAME in both experimental groups. Additionally, neither the basal and the EFS-induced NO release nor vasodilator response to the NO analogue DEA-NO were modified by LTPH. These results indicate that the participation of nitrergic innervation is also restored in LTPH. The fact that pre-incubation with CGRP-(8–37) did not alter vasoconstrictor response to EFS in SO confirms the non-participation of CGRP in aging [16], and indicates that, in contrast to STPH, the influence of sensory innervation is attenuated in LTPH.

Taken together, the present results indicate that the innervations of mesenteric arterial vessels may well participate in the development of the characteristic hyperdynamic circulation observed in STPH through the combined actions of a decrease in the influence of the adrenergic innervation and an increase in the influences of the nitrergic and sensory innervations. By contrast, we suggest that the influences of each type of innervation normalize under the conditions of LTPH.

AUTHOR CONTRIBUTION
Esther Sastre performed some of the experiments and statistical analyses. Gloria Balfagón performed some of the statistical analyses, discussed the results and wrote the paper. Elena Revuelta-López and Maria-Paz Nava performed the surgical techniques and the PP measurements. María-Ángeles Aller and Jaime Arias collaborated in the discussion of the results. Javier Blanco-Rivero performed some of the experiments and statistical analyses, discussed the results and wrote the paper.

FUNDING
This work was supported by the Ministerio de Ciencia e Innovación (MCINN) [grant number 2009-10374].

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