

RESEARCH PAPER

Rosuvastatin restored adrenergic and nitrenergic function in mesenteric arteries from obese rats

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BACKGROUND AND PURPOSE

We investigated whether high-fat diet (HFD)-induced obesity was associated with changed function of components of the mesenteric innervation (adrenergic, sensory and nitrenergic), the mechanisms involved and the possible effects of rosuvastatin on these changes.

EXPERIMENTAL APPROACH

Male Wistar rats were divided into three groups. (i) rats fed a standard diet (control group); (ii) rats fed a HFD (33.5% fat) for 7 weeks; and (iii) rats fed a HFD and treated with rosuvastatin (15 mg·kg⁻¹·day⁻¹) for 7 weeks. Segments of isolated mesenteric arteries were exposed to electric field stimulation (EFS) with or without tetrodotoxin, phentolamine, 7-nitroindazole (7NI) or N^o nitro-L-arginine methyl ester (L-NAME). Noradrenaline, ATP and NO release, and nNOS expression were also measured.

KEY RESULTS

EFS induced a greater frequency-dependent contraction in obese than in control rats. In HFD rats, phentolamine reduced contractions elicited by EFS, but noradrenaline release was greater and ATP release decreased. L-NAME and 7NI increased contractions to EFS in segments from control rats, but not in those from HFD rats. NO release and nNOS expression were lower in arterial segments from HFD rats than in control rats. All these changes in HFD rats were reversed by treatment with rosuvastatin.

CONCLUSIONS AND IMPLICATIONS

Neural control of mesenteric vasomotor tone was altered in HFD rats. Enhanced adrenergic and diminished nitrenergic components both contributed to increased vasoconstrictor responses to EFS. All these changes were reversed by rosuvastatin, indicating novel mechanisms of statins in neural regulation of vascular tone.

Abbreviations

7NI, 7 nitroindazole; CGRP, calcitonin gene-related peptide; DEA NO, diethylamine NONOate; EFS, electric field stimulation; HDL, high-density lipoprotein; HFD, high-fat diet; KHS, Krebs Henseleit solution; LDL, low-density lipoproteins; L-NAME, N^o nitro-L-arginine methyl ester; nNOS, neuronal nitric oxide synthase; PGI₂, prostaglandin I₂; TG, triglyceride; TTX, tetrodotoxin; VLDL, very low density lipoprotein

Introduction

Dietary factors, especially hypercaloric diets, are a determinant in the etiology of obesity in humans. Numerous studies in rodents have shown that chronic consumption of high-fat diets (HFDs)

induces increased gain in weight along with metabolic and cardiovascular alterations such as insulin resistance, dyslipidaemia, hypertension, cardiac haemodynamic disturbances and renal function impairment (Williams *et al.*, 2003; de las Heras *et al.*, 2009).

Accumulating experimental and clinical evidence indicates that obesity is associated with vascular function alterations. Endothelial dysfunction, due to altered NO release and metabolism, has been reported as participating in these vascular alterations (Javeshghani *et al.*, 2009; Belin de Chantemèle *et al.*, 2010; Miâdi-Messaoud *et al.*, 2010). The angiotensin II system also seems to be involved in obesity-associated alterations in both humans and rats (de las Heras *et al.*, 2009; Elmarakby and Imig, 2010; Katragadda and Arora, 2010; Rueda-Clausen *et al.*, 2010). The sympathetic nervous system seems to participate in the pathological cardiovascular and metabolic alterations associated with obesity (Prior *et al.*, 2010). However, certain controversial results have been reported and appear to be related to the specific vascular bed studied. The sympathetic nervous system is activated in human and experimental obesity produced by overfeeding, and has been proposed as one of the main mechanisms leading to the development of hypertension. This is probably attributable to the activation of the sympathetic outflow to the kidneys, and, perhaps, to insulin resistance (Straznicky *et al.*, 2008).

Vascular innervation plays a key role in the regulation of tone in certain vascular beds such as the mesenteric territory. Three components have been described as participating in nervous control of vascular tone of rat mesenteric artery: adrenergic, sensory and nitrergic (Kawasaki *et al.*, 1988; Li and Duckles, 1992; Boeckxstaens and Pelckmans, 1997; Marín and Balfagón, 1998). Alterations of the functional role of these components have been associated with several experimental and pathophysiological circumstances including ageing, hypertension and diabetes (Ferrer *et al.*, 2000b; 2001; 2003; Marín *et al.*, 2000; Minoves *et al.*, 2002; Hatanaka *et al.*, 2006; Iwatani *et al.*, 2008).

Drugs targeting the cardiovascular system such as adrenoceptor antagonists, angiotensin converting enzyme inhibitors (ACEI) and angiotensin AT₁ receptor antagonists (ARBs) have shown beneficial effects in metabolic and cardiovascular disorders associated with obesity through several mechanisms involving endothelial factors, inflammation and oxidative stress (de las Heras *et al.*, 2009; Elmarakby and Imig, 2010; Katragadda and Arora, 2010). Statins have also shown anti-inflammatory effects, as well as improvement in NO availability through hypolipidemic and pleiotropic mechanisms (Tan *et al.*, 2002; Gelosa *et al.*, 2007). However, the effects of statins on alterations of vascular innervation associated with obesity, have not been investigated. Thus, in the present study we aimed to investigate: (i) whether HFD-induced obesity in rats was associated with changes in the function of the

three components of mesenteric innervation, as well as the possible mechanisms involved; and (ii) the effects of a statin treatment on the possible functional mesenteric alterations observed in HFD-induced obesity.

Methods

Experimental procedure

All animal care and experimental procedures complied with the European Union guidelines on the ethical care of experimental animals, and US National Institutes of Health guidelines. The study was conducted with male Wistar rats (250 g; Harlan Ibérica SL, Barcelona, Spain). Animals were divided into three groups. (i) rats fed a standard diet for 7 weeks as a reference group for normal conditions (control); (ii) rats fed a HFD (33.5% fat) (Harlan Teklad #TD.03307, MN, USA) for 7 weeks (de las Heras *et al.*, 2009); and (iii) rats fed the same HFD (33.5% fat) and treated with rosuvastatin (15 mg·kg⁻¹ day) for 7 weeks. The dose of rosuvastatin was chosen from pilot studies from previous reports (Di Napoli *et al.*, 2005; Otto *et al.*, 2005; Schäfer *et al.*, 2005), where this drug showed a hypolipidemic effect in experimental models. Body weight was measured once a week. Food and water intake were determined throughout the experimental period.

After 7 weeks of treatment, the animals were killed; the first branch of the mesenteric artery was carefully dissected, cleaned of connective tissue and placed in Krebs-Henseleit solution (KHS, in mmol·L⁻¹: NaCl 115, CaCl 2 2.5, KCl 4.6, KH₂PO₄ 1.2, MgSO₄·7H₂O 1.2, NaHCO₃ 25, glucose 11.1, Na₂EDTA 0.03) at 4°C. Body weight at the end of the experiment was normalized to tibia length, because of the important body weight differences between HFD rats and controls. Tibia length is used to compare parameters in animals where the main variable in the model is body weight (Khamaisi *et al.*, 2007; de las Heras *et al.*, 2009; Xu *et al.*, 2009).

Lipid profile

Plasma concentrations of total cholesterol, triglycerides (TG) and HDL-cholesterol were determined using specific quantitative sandwich enzyme immunoassay (R&D Systems, Minneapolis, MN, USA). As the calculation of LDL cholesterol could underestimate the lipid alterations in situations where TG are elevated, as in our model of obesity, non-HDL cholesterol was calculated. Non-HDL cholesterol (which includes both LDL and VLDL cholesterol) is considered more appropriate in conditions of high TG.

Vascular reactivity

The method used for isometric tension recording has been described in full elsewhere (Nielsen and Owman, 1971). Two parallel stainless steel pins were introduced through the lumen of the vascular segment: one was fixed to the bath wall, and the other connected to a force transducer (Grass FTO3C; Quincy, MA, USA); this was connected in turn to a model 7D Grass polygraph. For electric field stimulation (EFS) experiments, segments were mounted between two platinum electrodes 0.5 cm apart and connected to a stimulator (Grass, model S44) modified to supply the appropriate current strength. Segments were suspended in an organ bath containing 5 mL of KHS at 37°C continuously bubbled with a 95% O₂-5% CO₂ mixture (pH of 7.4). Experiments were performed in endothelium-denuded segments in order to eliminate complex interferences of vasoactive substances released from endothelial cells [such as NO, endothelin-1, prostacyclin (PGI₂), angiotensin II] as we have previously demonstrated (Ferrer *et al.*, 1992; 2000a; Iwatani *et al.*, 2008). Interrelationships among the many endothelium-derived agents and the components of mesenteric innervation would make the study extremely complicated. In addition, endothelium denudation avoids possible actions by different drugs used on endothelial cells that could lead to misinterpretation of the results. Endothelium was removed by gently rubbing the luminal surface of the segments with a thin wooden stick. The segments were subjected to a tension of 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⁻¹ KCl to check their functional integrity. Endothelium removal did not alter the contractions elicited by 75 mmol·L⁻¹ KCl. After a washout period, the absence of vascular endothelium was tested by the inability of 10 μmol·L⁻¹ ACh to relax segments precontracted with 1 μmol·L⁻¹ noradrenaline.

Two consecutive frequency-response curves to EFS (1, 2, 4, 8 and 16 Hz) were performed in mesenteric segments from all experimental groups. The parameters used for EFS were 200 mA, 0.3 ms, 1–16 Hz, for 30 s with a one-minute interval between each stimulus, which is the time required to recover basal tone. These parameters were used in previous studies due to their similarity to physiological conditions (Marín and Balfagón, 1998). A washout period of at least 1 h was necessary to avoid desensitization between consecutive curves.

To evaluate whether EFS-induced contractile response had a neural origin, the blocker for nerve impulse propagation, tetrodotoxin (TTX;

0.1 μmol·L⁻¹) was added to the bath 30 min before EFS.

To determine the contribution of adrenergic innervation to EFS-induced responses in segments from control, HFD and HFD + rosuvastatin rats, 1 μmol·L⁻¹ phentolamine, a non-specific α-adrenoceptor antagonist (Marín and Balfagón, 1998), was added to the bath 30 min before performing the frequency-response curve.

In order to analyse the possible participation of sensory innervation on EFS-induced response, 0.5 μmol·L⁻¹ the fragment of calcitonin-gene related peptide (CGRP 8-37), a CGRP receptor antagonist, was added to the bath 30 min before performing the frequency-response curve in segments from all of the experimental groups.

To determine the participation of NO in EFS-induced response in all groups of rats, the non-selective NO synthase (NOS) inhibitor, N^ω nitro-L-arginine methyl ester (L-NAME; 0.1 μmol·L⁻¹), 1 μmol·L⁻¹ 1400W, a selective inhibitor of inducible NOS inhibitor, or 0.1 mmol·L⁻¹ 7-nitroindazole (7NI), a specific inhibitor of neuronal NOS, was added to the bath 30 min before performing the frequency-response curve.

The vasoconstrictor response to exogenous noradrenaline (1 nmol·L⁻¹–10 μmol·L⁻¹) in presence/absence of L-NAME (0.1 μmol·L⁻¹), and 7NI (0.1 mmol·L⁻¹) were tested in arteries from all experimental groups. In addition, the vasodilator response to NO donor diethylamine NONOate (DEA-NO; 0.1 nmol·L⁻¹–0.1 mmol·L⁻¹) was tested in arteries from all experimental groups. Artery function was checked after the entire experiment by exposing arteries to 75 mmol·L⁻¹ KCl.

Noradrenaline, CGRP, and ATP release

Endothelium-denuded segments from control, HFD and HFD + rosuvastatin rats were preincubated for 30 min in 5 mL of KHS at 37°C and continuously gassed with a 95% O₂-5% CO₂ mixture (stabilization period). This was followed by two washout periods of 10 min in a bath of 0.4 mL of KHS. Then, the medium was collected to measure basal release. Once the bath was refilled, arteries were subjected to cumulative 30-second EFS periods at 1, 2, 4, 8 and 16 Hz at 1 min intervals. At the end of EFS stimulation, the medium was collected to measure neurotransmitter release. Samples were frozen in liquid nitrogen and stored at -70°C until the assay was performed. To measure the release of noradrenaline, CGRP or ATP we used a noradrenaline research EIA (Labor Diagnostica Nord, GmbH & Co., KG, Nordhon, Germany), a rat CGRP enzyme immunoassay kit (Spibio, Bertin Group, Montigny le Bretonneux, France), or an ATP Colorimetric/

Fluorometric Assay kit (Abcam, Cambridge, UK). Assays were performed following the manufacturer's instructions. Results were expressed as ng noradrenaline·mL⁻¹ per mg of tissue, pg CGRP·mL⁻¹ per mg tissue, or nmol ATP·mg⁻¹ tissue.

Release of NO

NO release was measured as previously described (del Campo *et al.*, 2009). Endothelium-denuded segments from control, HFD and HFD + rosuvastatin rats were subjected to a resting tension of 0.5 g as indicated for reactivity experiments. After an equilibration period of 60 min in HEPES buffer (in mmol·L⁻¹: NaCl 119; HEPES 20; CaCl₂ 1.2; KCl 4.6; MgSO₄ 1; KH₂PO₄ 0.4; NaHCO₃ 5; glucose 5.5; Na₂HPO₄ 0.15; pH 7.4) at 37°C, arteries were incubated with the fluorescent probe 4,5-diaminofluorescein (DAF-2; 2 μmol·L⁻¹) for 45 min. Then, the medium was collected to measure basal NO release. Once the organ bath was refilled, 30 s cumulative EFS periods at 1, 2, 4, 8 and 16 Hz at 1 min intervals were applied. At the end of EFS stimulation, the medium was collected to measure EFS-induced NO release. The fluorescence of the medium was measured at room temperature using a spectrofluorimeter (LS50 Perkin Elmer instruments, Waltham, MA, USA, FL WINLAB Software) with excitation wavelength set at 492 nm and emission wavelength at 515 nm.

The EFS-induced NO release was calculated by subtracting basal NO release from that evoked by EFS. Also, blank samples were collected in the same way as from segment-free medium in order to correct for background emission. To confirm the neural origin of NO, some assays were performed in the presence of 0.1 mmol·L⁻¹ 7-NI or 0.1 μmol·L⁻¹ TTX. The amount of NO released was expressed as arbitrary units·mg⁻¹ tissue.

Western blot analysis of neuronal NOS

For Western blot analysis of nNOS expression, endothelium-denuded mesenteric segments from all experimental groups were homogenized in a boiling buffer composed of 1 mM sodium vanadate, 1% sodium dodecyl sulfate (SDS), and 0.01 M pH 7.4 Tris-HCl. Homogenates containing 30 μg protein were electrophoretically separated on a 7.5% SDS-polyacrylamide gel, and then transferred to a polyvinyl difluoride membrane (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 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 solution (100 mM, 0.9% w/v NaCl, 0.1% SDS) with 5% powdered fat-free milk before

being incubated overnight at 4°C with mouse monoclonal antibody against nNOS (1:2000 dilution, Transduction laboratories). After washing, the membrane was incubated with anti-mouse horseradish peroxidase-conjugated immunoglobulin G antibody (Amersham International Plc, Little Chalfont, UK). The membrane was thoroughly washed and the immunocomplexes were then detected using an enhanced horseradish peroxidase/luminol chemiluminescence system (ECL Plus, Amersham International Plc) and subjected to autoradiography (Hyperfilm ECL, Amersham International Plc). Signals on the immunoblot were quantified using a computer program (NIH Image 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 anti α-actin antibody (1:2000 dilution, Sigma, Madrid, Spain). Rat brain homogenates were used as positive control.

Data analysis

The responses elicited by EFS and noradrenaline were expressed as a percentage of the initial contraction elicited by 75 mmol·L⁻¹ KCl for comparison between experimental groups. The relaxation induced by DEA-NO was expressed as a percentage of the initial contraction elicited by noradrenaline. Results are given as mean + SEM. Statistical analysis was done by comparing the curve (1 to 16 Hz) obtained in the presence of the different substances with the control curve by means of repeated measure analysis of variance (ANOVA) followed by Bonferroni's test.

For the experiments on NO, CGRP, ATP and noradrenaline release, the statistical analysis was done using Student's *t*-test for unpaired experiments. *P* < 0.05 was considered significant.

Materials

L-noradrenaline hydrochloride, ACh chloride, CGRP, CGRP 8-37, diethylamine NONOate diethylammonium salt, TTX, 1400W, L-NAME hydrochloride, 7-nitroindazole, phentolamine, tempol and DAF-2 were purchased from Sigma-Aldrich. Stock solutions (10 mmol·L⁻¹) 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, and 7NI and tempol, which were dissolved in DMSO. The final DMSO concentration did not alter any of the responses in the current studies. These solutions were stored at -20°C and appropriate dilutions were made in KHS on the day of the experiment. Drug and receptor nomenclature follows Alexander *et al.* (2009).

Table 1

Final values of body weight, food intake and biochemical parameters

	Control	HFD	HFD + rosuvastatin
Body weight (g·cm ⁻¹)	321 ± 13	423 ± 14*	437 ± 8.3*
Food intake (g)	6.94 ± 0.36	3.41 ± 0.27*	3.59 ± 0.4*
Total cholesterol (mg·L ⁻¹)	458 ± 34	470 ± 35	418 ± 40
High-density lipoprotein (HDL) cholesterol (mg·L)	356 ± 16	358 ± 22	340 ± 24
Non-HDL cholesterol (mg·L ⁻¹)	102 ± 20	152 ± 31	78 ± 19#
Triglycerides (mg·L ⁻¹)	508 ± 35	1072 ± 9*	710 ± 52#

At the end of the experimental period of 7 weeks, we measured body weight (normalised to tibia length and expressed as g·(cm tibia)⁻¹, plasma total cholesterol: HDL-cholesterol, non-HDL cholesterol and triglycerides, in rats fed a standard diet (Control), high-fat diet (HFD) and rats fed a high-fat diet treated with rosuvastatin at 15 mg·kg⁻¹·day⁻¹ (HFD + rosuvastatin) for 7 weeks. Data shown are means ± SEM.

**P* < 0.05 versus control; #*P* < 0.05 versus HFD.

Results

General characteristics

From the first week onwards, body weight gain was significantly higher in rats fed a HFD compared with rats fed a standard diet (Figure 1). The difference in body weight at the end of experiment was 27% (Table 1). Treatment with rosuvastatin did not affect body weight in HFD rats. Food intake was lower (*P* < 0.05) in HFD rats compared with controls throughout the experiment. Treatment with rosuvastatin did not modify food intake in HFD rats (Table 1).

Lipid profile

Plasma total cholesterol and HDL-cholesterol levels were comparable in the three groups. Non-HDL cholesterol levels were comparable in control and HFD rats. However, treatment with rosuvastatin reduced (*P* < 0.05) non-HDL cholesterol levels in HFD rats.

TG levels were higher (*P* < 0.05) in rats given a HFD than in controls, and these elevated levels were restored to normal values by treatment with rosuvastatin (Table 1).

Vascular reactivity

Vasoconstrictor response induced by 75 mmol·L⁻¹ KCl was similar in segments from all groups of rats (control, 1028 ± 64 mg; HFD, 912 ± 60; HFD + rosuvastatin, 943 ± 95 mg; *P* > 0.05; *n* = 10 per group). No differences were found between KCl vasoconstrictor response at the beginning and at the end of the experiment (control, 1019 ± 85 mg; HFD, 928 ± 64; HFD + rosuvastatin, 979 ± 97 mg; *P* > 0.05; *n* = 10 per group).

EFS induced a frequency-dependent contraction, which was greater (*P* < 0.05) in HFD than in control rats (Figure 2). Treatment with rosuvastatin reduced EFS-induced contractions to a level similar to that in

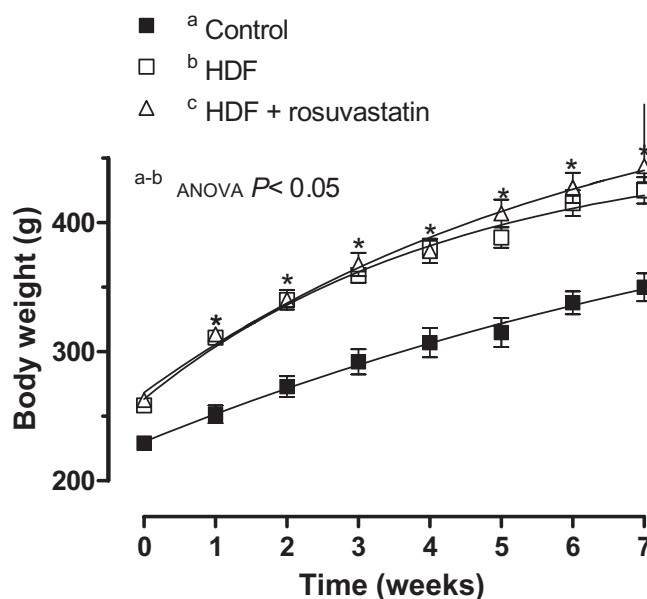


Figure 1

Weekly increases in body weight in control, high-fat diet (HFD) and HFD + rosuvastatin rats. Results are expressed as mean ± SEM *n* = 6–10 animals per group. **P* < 0.05 HFD versus control rats for each week (Bonferroni test).

control rats (Figure 2). EFS-induced contractions were practically abolished in segments from all experimental groups by the nerve impulse propagation blocker, TTX (0.1 μmol·L⁻¹; Figure 2).

The contraction elicited by EFS was significantly reduced by the non-selective α-adrenoceptor antagonist, phentolamine (1 μmol·L⁻¹), in segments from all groups of rats, suggesting noradrenaline participation. The decrease was higher in HFD rats than in controls, and was similar to that observed in HFD + rosuvastatin rats (Figure 3).

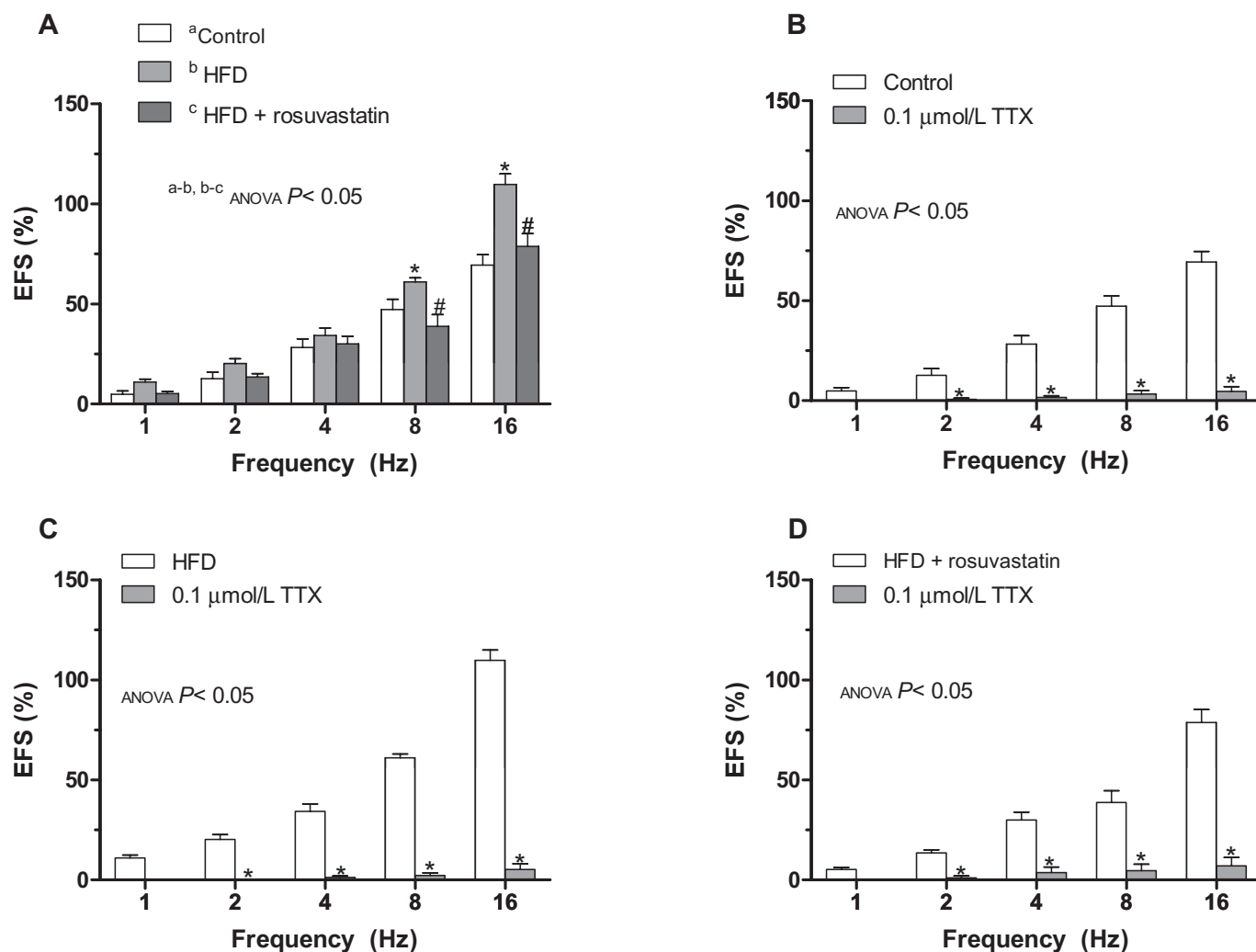


Figure 2

(A) Vasoconstrictor response to electric field stimulation (EFS) in segments from control, high-fat diet (HFD) and HFD + rosuvastatin rats. * $P < 0.05$ versus control animals for each frequency (Bonferroni test). # $P < 0.05$ versus HFD animals for each frequency (Bonferroni test). Effect of $0.1 \mu\text{mol}\cdot\text{L}^{-1}$ TTX on the vasoconstrictor response induced by EFS in segments from control (A), HFD (B) and HFD + rosuvastatin (C) rats. Results (mean \pm SEM) are expressed as a percentage of previous contraction elicited by KCl. $n = 6$ – 10 animals per group. * $P < 0.05$ versus conditions without specific inhibitor for each frequency (Bonferroni test).

The contractile response induced by exogenous noradrenaline ($0.1 \text{ nmol}\cdot\text{L}^{-1}$ – $10 \mu\text{mol}\cdot\text{L}^{-1}$) was similar in mesenteric segments from all experimental groups (Figure 3).

The vasodilator response induced by exogenous CGRP was similar in all experimental groups (Figure 4). The CGRP receptor antagonist CGRP 8-37 ($0.5 \mu\text{mol}\cdot\text{L}^{-1}$) did not modify the contractile response induced by EFS in any of the experimental groups, indicating that the sensory nerves did not contribute to the observed effects (Figure 4).

The contraction induced by EFS was increased by preincubation with L-NAME and 7NI in segments

from control and HFD + rosuvastatin rats, but not in HFD rats (Figure 5). The effect in presence of L-NAME was higher than that observed in presence of 7NI. The specific iNOS inhibitor 1400W did not modify EFS-induced contraction in any of the experimental groups (Figure 5). In noradrenaline-precontracted mesenteric segments ($995 \pm 126 \text{ mg}$), DEA-NO ($0.1 \text{ nmol}\cdot\text{L}^{-1}$ – $0.1 \text{ mmol}\cdot\text{L}^{-1}$) induced a similar concentration-dependent relaxation in segments from all of the experimental groups (Figure 6).

In addition, 7NI decreased noradrenaline-induced contraction in mesenteric segments from all of the experimental groups to a similar extent,

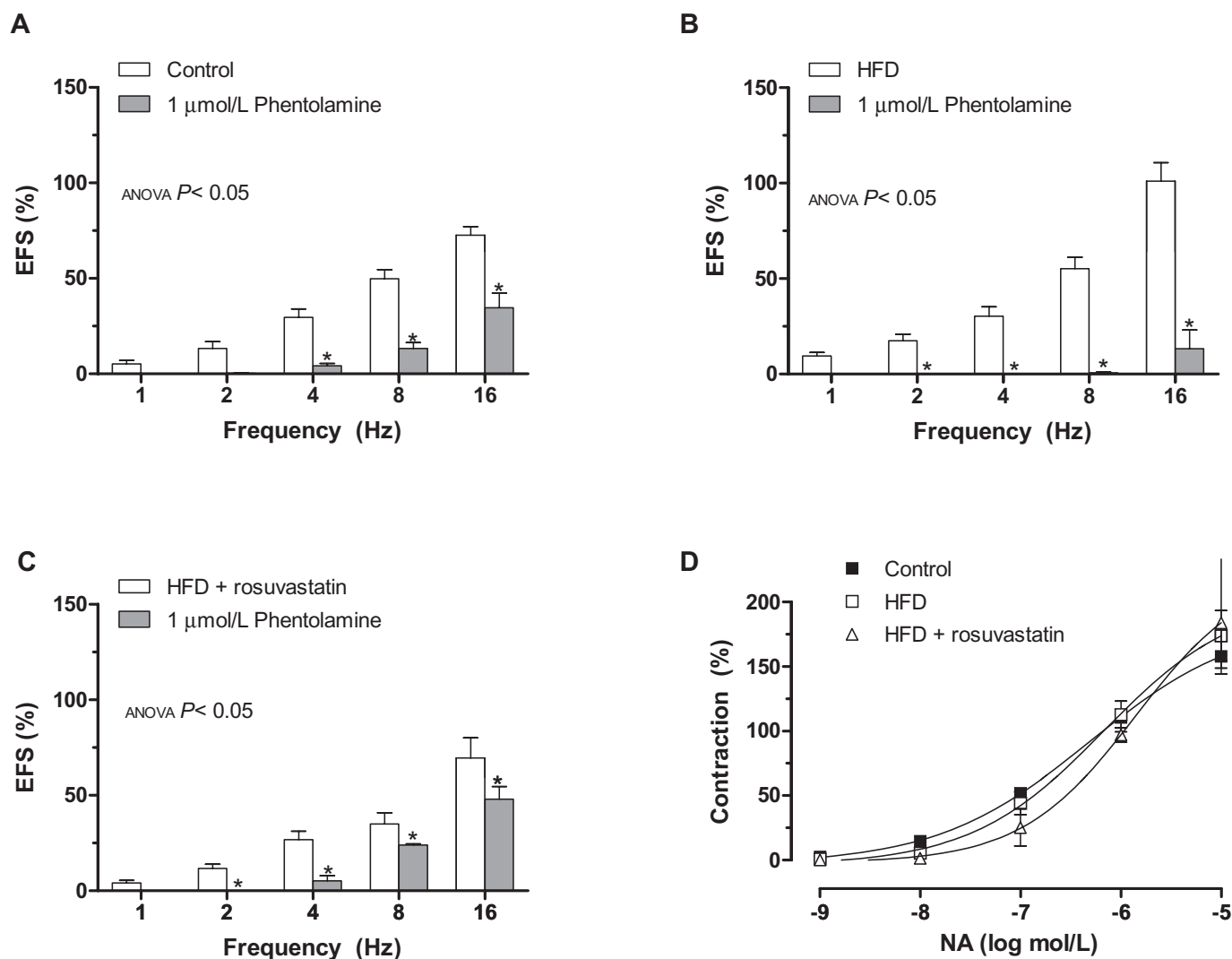


Figure 3

Effect of preincubation with $1 \mu\text{mol}\cdot\text{L}^{-1}$ phentolamine on the vasoconstrictor response induced by electric field stimulation (EFS) in mesenteric segments from control (A), high-fat diet (HFD) (B) and HFD + rosuvastatin (C) rats. (D) Vasoconstrictor response to exogenous noradrenaline in segments from control, HFD and HFD + rosuvastatin rats. Results (mean \pm SEM) are expressed as a percentage of previous contraction elicited by KCl. $n = 6\text{--}10$ animals per group. * $P < 0.05$ versus conditions without specific inhibitor for each frequency (Bonferroni test).

whereas it was not modified by preincubation with L-NAME (Figure 7, Table 2).

Noradrenaline release

Both basal and EFS-induced noradrenaline releases were greater in HFD than in control rats (Figure 8A). Treatment with rosuvastatin reduced noradrenaline release to control levels (Figure 8A).

CGRP release

Both basal and EFS-induced CGRP releases were greater in HFD than in control rats (Figure 8B). Treatment with rosuvastatin reduced CGRP release to control levels (Figure 8B).

ATP release

Basal ATP release was similar in mesenteric segments from, control, HFD and rosuvastatin-treated HFD rats (Figure 8C). EFS induced ATP release segments in control and rosuvastatin-treated rats, but not in HFD animals (Figure 8C).

NO release

Both basal and EFS-induced release of NO were lower in HFD than in control mesenteric segments (Figure 8D). Treatment with rosuvastatin increased both basal and EFS-induced NO release to control levels (Figure 8D). In addition, 7NI ($0.1 \text{ mmol}\cdot\text{L}^{-1}$)

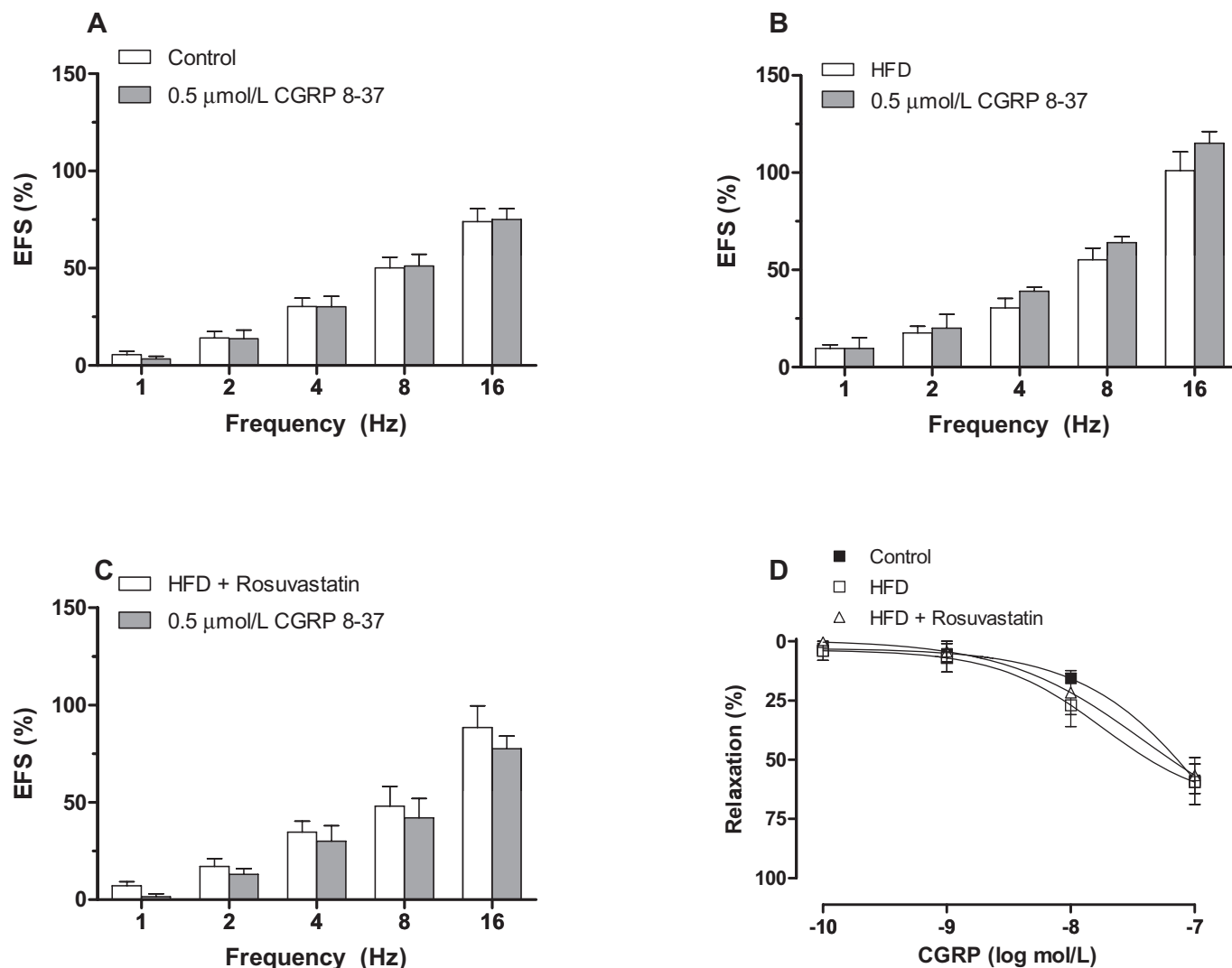


Figure 4

Effect of preincubation with $0.5 \mu\text{mol}\cdot\text{L}^{-1}$ CGRP 8-37 on the vasoconstrictor response induced by electric field stimulation (EFS) in mesenteric segments from control (A), high-fat diet (HFD) (B) and HFD + rosuvastatin (C) rats. Results (mean \pm SEM) are expressed as a percentage of previous contraction elicited by KCl. $n = 6-10$ animals per group. (D) Vasodilator response to exogenous CGRP in segments from control, HFD and HFD + rosuvastatin rats. Results (mean \pm SEM) are expressed as a percentage of previous contraction elicited by noradrenaline. $n = 6-10$ animals per group.

and TTX ($0.1 \mu\text{mol}\cdot\text{L}^{-1}$) practically abolished EFS-induced NO release in arteries from both groups (Figure 9A–C).

*n*NOS protein expression

HFD-rats presented a reduced ($P < 0.05$) nNOS protein expression compared with controls. Treatment with rosuvastatin partially prevented ($P < 0.05$) this reduction (Figure 9D).

Discussion

The main findings of the present work show that HFD-induced obesity produces alterations of the

adrenergic and nitrergic innervation of rat mesenteric artery, which are restored by treatment with rosuvastatin.

In the three experimental groups, EFS produced a frequency-dependent contraction, which was abolished in the presence of TTX, indicating that the vasomotor responses are the result of neurotransmitter release from perivascular nerves. HFD produced an increased response to EFS, which was not attributable to changes in the intrinsic contractile machinery as demonstrated by the similar vasoconstrictor response to KCl in the three experimental groups. The enhanced response to EFS was normalized by treatment with rosuvastatin. To our knowledge, this is the first study to show this novel effect

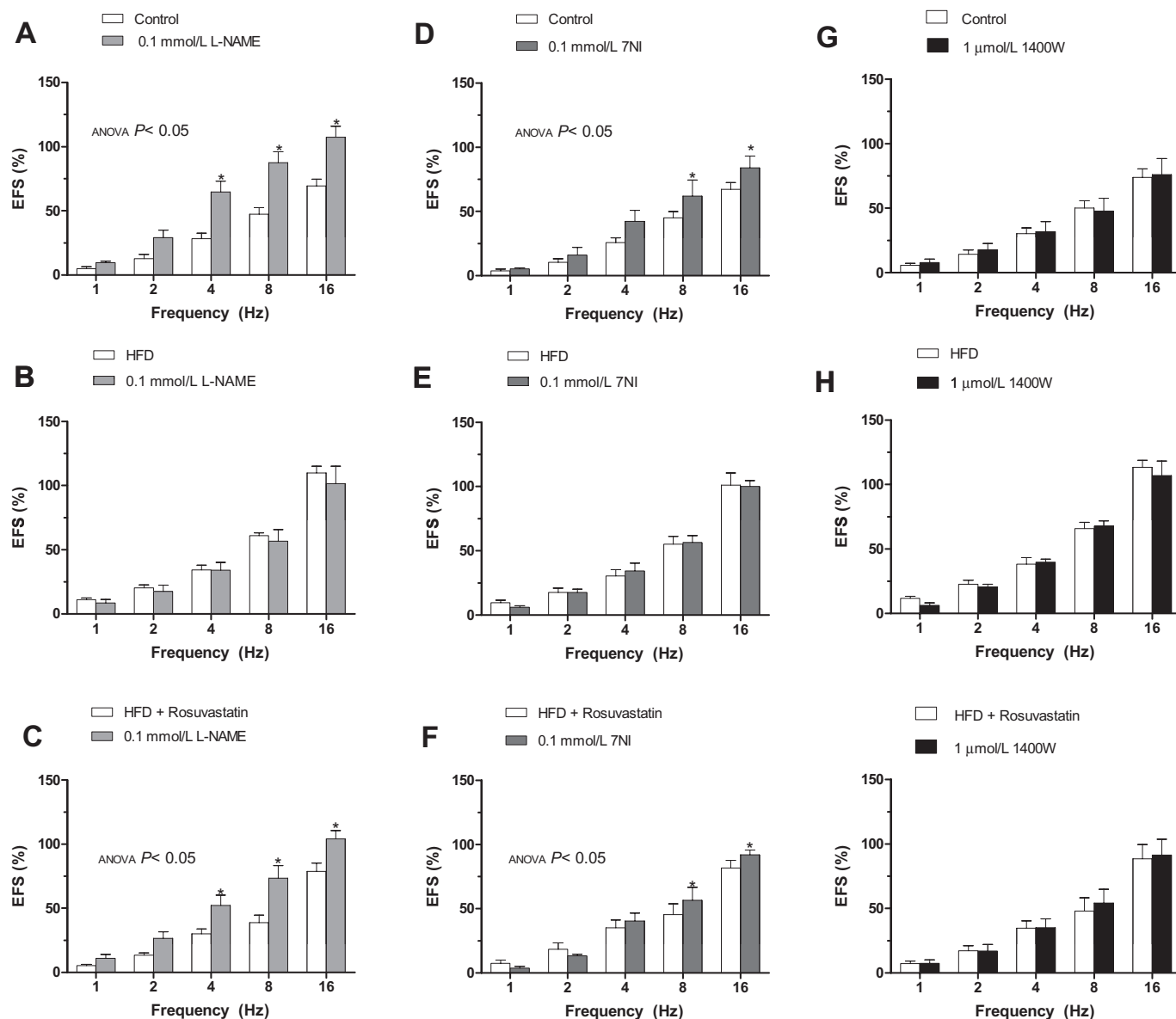


Figure 5

Effect of preincubation with 0.1 mmol·L⁻¹ N^ω nitro-L-arginine methyl ester (L-NAME) (A–C), 0.1 mmol·L⁻¹ 7-NI (D–F) or 1 μmol·L⁻¹ 1400W (G–I) on the vasoconstrictor response induced by electric field stimulation (EFS) in mesenteric segments from control (A,D,G), high-fat diet (HFD) (B,E,H) and HFD + rosuvastatin (C,F,I) rats. Results (mean ± SEM) are expressed as a percentage of previous contraction elicited by KCl. *n* = 6–10 animals per group. **P* < 0.05 versus conditions without specific inhibitor for each frequency (Bonferroni test).

of statins in the regulation of vascular innervation. The decrease in non-HDL cholesterol levels could be attributed to the observed effects of the statin, although other well-known described effects of statins, such as antioxidant and anti-inflammatory effects, might have contributed to the vascular effects of rosuvastatin (Devaraj *et al.*, 2006; Cachofeiro *et al.*, 2008; Chen *et al.*, 2009).

As mentioned in the Introduction, adrenergic, sensory and nitrergic innervation are involved in the vasomotor response to EFS in rat mesenteric artery (Kawasaki *et al.*, 1988; Li and Duckles, 1992;

Boeckxstaens and Pelckmans, 1997; Marín and Balfagón, 1998). The fact that EFS-induced net response is a contraction suggests a predominant role of the adrenergic component. To explore the participation of adrenergic component in the enhanced response to EFS in obese rats, the effects of the non-selective α -adrenergic antagonist, phentolamine, were analysed. The reduction of contractile response in presence of phentolamine was higher in HFD rats than in controls, confirming a higher participation of adrenergic innervation in arteries from obese rats than those from in normal

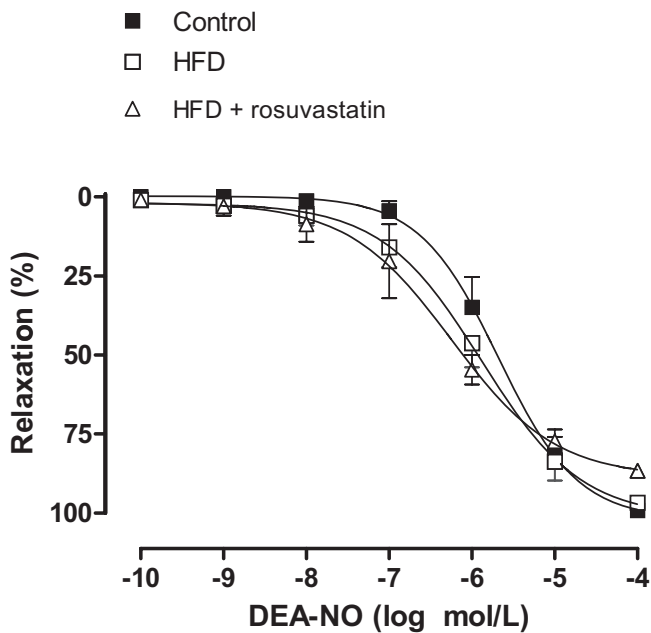


Figure 6

Relaxation to diethylamine NONOate (DEA-NO) in segments from control, high-fat diet (HFD) and HFD + rosuvastatin rats. Results (mean \pm SEM) are expressed as a percentage of previous contraction elicited by noradrenaline. $n = 6$ – 10 animals per group.

rats. This is in agreement with previous results showing adrenergic hyperactivity in obesity (Scherer *et al.*, 1994; Straznický *et al.*, 2008). The observed adrenergic effect in HFD rats could be mediated by either an enhanced response to noradrenaline or an enhanced release of noradrenaline from the nervous terminal. Contractile response to noradrenaline was comparable in the three experimental groups, whereas basal and EFS-induced release of noradrenaline was higher in obese rats. Thus, the observed enhanced adrenergic participation in HFD rats seems to be due to an increased release of noradrenaline. Furthermore, treatment with rosuvastatin restored noradrenaline release and thus, the participation of adrenergic component in vasoconstrictor response to EFS. It should also be mentioned that in the control group and in the HFD + rosuvastatin group, there was a substantial response to activation of the sympathetic nerves that was phentolamine resistant. The most likely transmitter for this response is ATP. In fact, ATP release was higher in normal and HFD + rosuvastatin rats. The results showing noradrenaline and ATP release confirm the coexistence of different mechanisms modulating sympathetic activation. This novel effect of statins in the regulation of vascular adrenergic innervation could have func-

tional relevance, in addition to its well-known effects on lipid metabolism and atherosclerotic mechanisms.

Although the present results confirm the major role of the adrenergic system in the elevation of mesenteric vascular tone in obesity, the participation of other neural components cannot be ruled out. In certain experimental conditions, such as hyperaldosteronism and hypertension, alterations in sensory function play a key role in the regulation of mesenteric vascular tone (Balfagón *et al.*, 2004; del Campo *et al.*, 2009). Exogenous CGRP produced a mild vasodilatory response, which was comparable in the three groups. However, release of this peptide was higher in HFD rats than in normal, and was restored by rosuvastatin treatment. To analyse the functional role of sensory innervation in obese rats, the effect of the CGRP receptor antagonist, CGRP 8-37, was studied in the constrictor response to EFS. No differences were observed between normal and HFD rats in presence or absence of the antagonist. This indicates that, apart from increased CGRP release in obese rats, the sensory innervation did not participate in the response to EFS in HFD rats. This lack of effect may suggest that the amount of CGRP released is not enough to produced any vasomotor response. As expected, rosuvastatin did not show any additional effect on the unaltered response to EFS in the presence of CGRP 8-37, although the statin restored CGRP release from mesenteric artery.

Several studies have already demonstrated the participation of NO from iNOS in pro-inflammatory states (Guzik *et al.*, 2003). As obesity has been considered as a state of low grade inflammation, we aimed to evaluate the potential participation of iNOS in the release of NO induced by EFS. However, preincubation with iNOS inhibitor 1400W did not modify vasoconstrictor response to EFS, indicating that iNOS is not involved in the different observed vascular responses in HFD rats. We previously showed that EFS stimulation produced neural release of NO (Ferrer *et al.*, 2004; Xavier *et al.*, 2004). Consequently, we studied the effects of the non-specific NOS inhibitor, L-NAME, or the specific nNOS inhibitor, 7NI, in the enhanced response to EFS in HFD rats. Normal but not HFD rats showed an enhanced response to EFS in presence of 7NI and L-NAME, where the effect of the latter was more marked than that of 7NI, probably due to the fact that 7NI also decreases the vasoconstrictor response to noradrenaline. This indicates that the participation of neural NO in the control of vasomotor response in the mesenteric artery is abolished in obese rats. This reduced participation of NO in HFD rats could be due to a reduction in NO production or

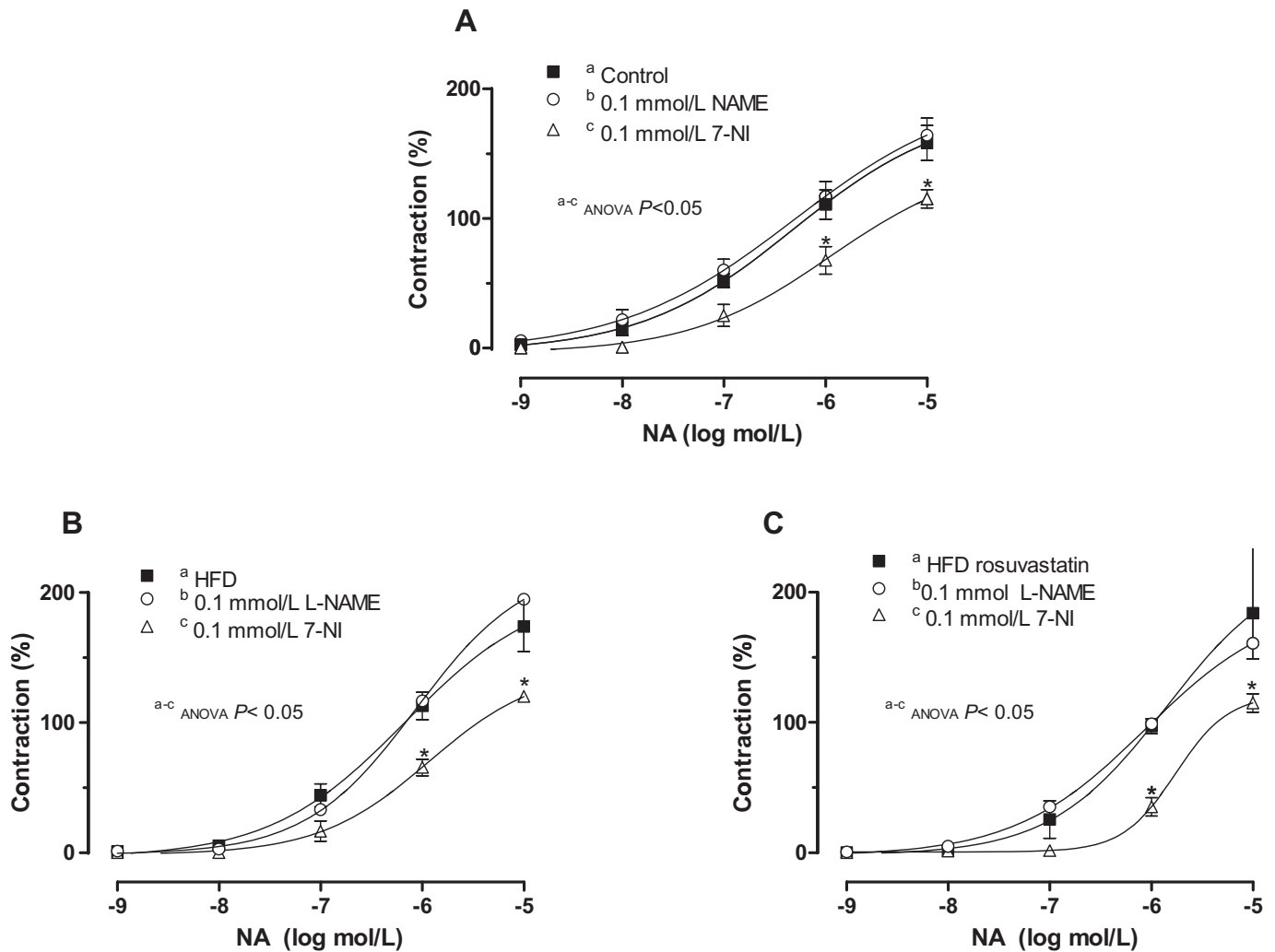


Figure 7

Effect of preincubation with 0.1 mmol·L⁻¹ N^o nitro-L-arginine methyl ester (L-NAME) or 0.1 mmol·L⁻¹ 7-NI on the vasoconstrictor response elicited by exogenous noradrenaline in mesenteric segments from control (A), high-fat diet (HFD) (B) and HFD + rosuvastatin (C) rats. Results (mean ± SEM) are expressed as a percentage of previous contraction elicited by KCl. $n = 6-10$ animals per group. * $P < 0.05$ versus conditions without specific inhibitor for each frequency (Bonferroni test).

Table 2

Effect of preincubation with L-NAME (0.1 mmol·L⁻¹) or 7-nitroindazole (7NI; 0.1 mmol·L⁻¹) on the vasoconstrictor responses to noradrenaline (Emax and pEC₅₀) in mesenteric segments from control, HFD and HFD + rosuvastatin rats

	Control Emax	pEC ₅₀	HFD Emax	pEC ₅₀	HFD + rosuvastatin Emax	pEC ₅₀
Untreated	204.0 ± 38.8	6.17 ± 0.31	204.0 ± 39.8	6.17 ± 0.31	225.5 ± 85.2	5.85 ± 0.53
L-NAME	200.6 ± 18.2	6.31 ± 0.42	222.1 ± 43.5	6.06 ± 0.28	192.7 ± 32.7	6.06 ± 0.26
7NI	141.7 ± 12.3*	5.93 ± 0.11	141.7 ± 12.3*	5.93 ± 0.12	121.2 ± 24.2*	5.76 ± 0.29

Results are expressed as mean ± SEM. Six to ten animals in each group.

* $P < 0.05$ versus untreated rats.

Emax, maximal effect; HFD, high-fat diet; pEC₅₀, -log EC50; L-NAME, ^o nitro-L-arginine methyl ester;

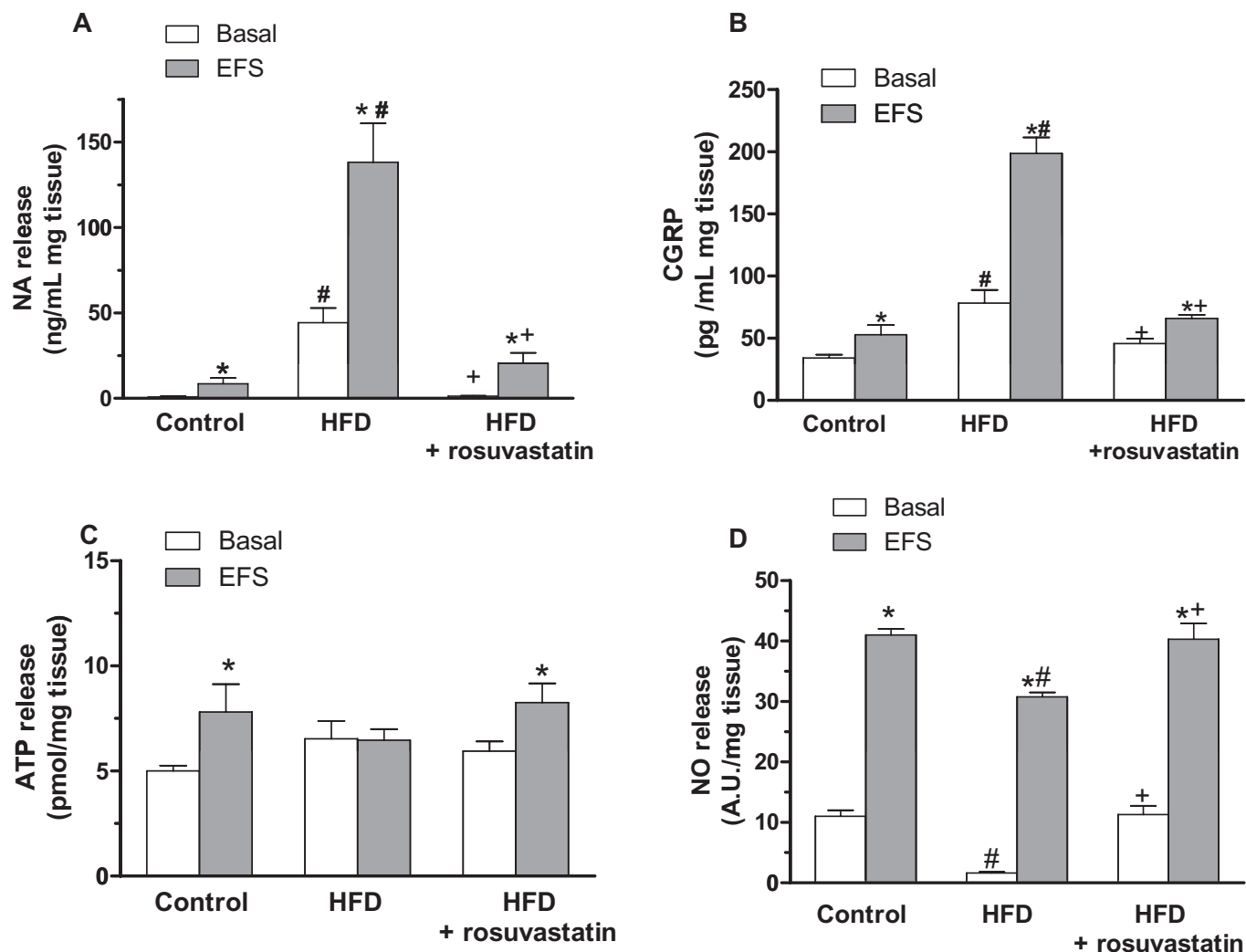


Figure 8

Basal and electric field stimulation (EFS)-induced noradrenaline (A), calcitonin gene-related peptide (CGRP) (B), ATP (C) and nitric oxide (NO) (D) release in segments from control, high-fat diet (HFD) and HFD + rosuvastatin rats. Results (mean \pm SEM) are expressed as ng noradrenaline·mL⁻¹ mg tissue, pg CGRP·mL⁻¹ mg tissue, pmol ATP·mg⁻¹ tissue and AU·mg⁻¹ tissue. **P* < 0.05 versus basal; #*P* < 0.05 versus control; +*P* < 0.05 versus HFD. *n* = 4–6 animals per group.

a diminished response to this agent in vascular smooth muscle. Responses to the NO donor DEA-NO were comparable in the three groups, suggesting that a different mechanism was responsible for the observed effects. Basal and stimulated NO release was reduced in obese rats, and almost abolished in presence of TTX, indicating that the diminished participation of nitrenergic innervation in obese rats is due to a reduction of NO release. As previously suggested in normal rats (Hatanaka *et al.*, 2006) NO released from perivascular nerves, inhibits neurogenic noradrenaline release, modulating adrenergic neurotransmission. This effect could also be valid in obese rats. Therefore, it could be proposed that the observed enhanced contractile response in mesenteric segments from HFD rats is the result of,

at least, two effects: an increased participation of adrenergic innervation, along with a reduction in the nitrenergic component. This latter notion was confirmed by the diminished nNOS expression observed in HFD rats. Treatment with rosuvastatin restored nNOS expression, as previously reported for other NOS isoforms (Di Napoli *et al.*, 2005; Landsberger *et al.*, 2005), and NO release, and consequently the participation of nitrenergic innervation in the response to EFS in mesenteric arteries from obese rats. Restoring nitrenergic function is a novel effect of rosuvastatin, further supporting the importance of statins in the regulation of vascular innervation. Furthermore, the different patterns of noradrenaline, ATP, CGRP and NO release also support the complex interactions modulating

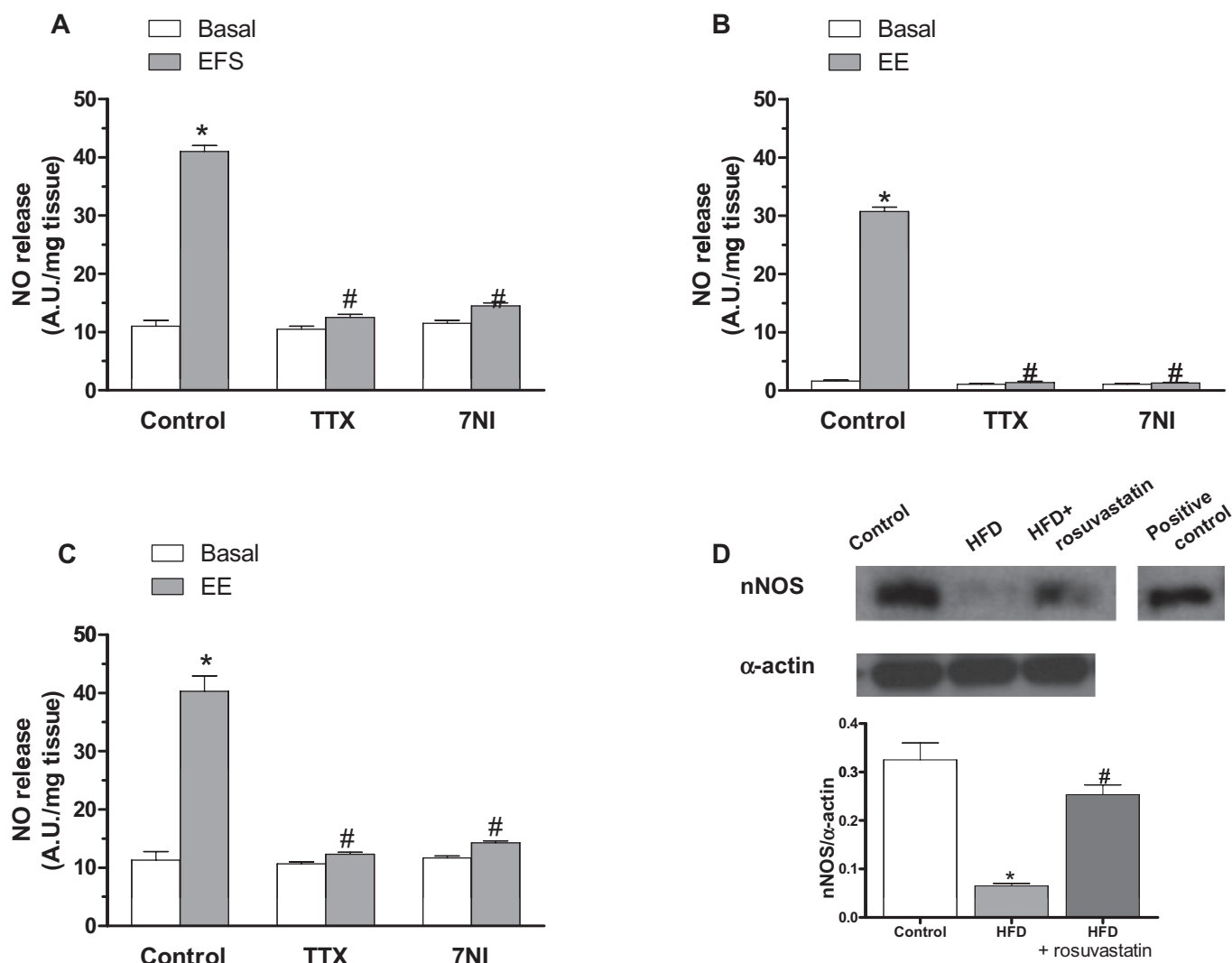


Figure 9

Effect of preincubation with $0.1 \mu\text{mol}\cdot\text{L}^{-1}$ tetrodotoxin (TTX) or $0.1 \text{mmol}\cdot\text{L}^{-1}$ 7-nitroindazole (7NI) on basal and electric field stimulation (EFS)-induced NO release in segments from control (A), high-fat diet (HFD) (B) and HFD + rosuvastatin (C) rats. Results (mean \pm SEM) are expressed as AU mg^{-1} tissue. * $P < 0.05$ versus basal; # $P < 0.05$ versus control. $n = 4\text{--}6$ animals per group. (D) Expression of nNOS in segments from control, HFD and HFD + rosuvastatin rats. The blot is representative of four separate segments from each group. Lower panel shows densitometric analysis of nNOS expression. Results (mean \pm SEM) are expressed as the ratio of the signal obtained for nNOS and that obtained for α -actin. * $P < 0.05$ versus basal; # $P < 0.05$ versus control; + $P < 0.05$ versus HFD.

mesenteric innervation (Dunn *et al.*, 1999; Brock and Tan, 2004).

Finally, it should be mentioned that the presence of endothelium could affect the observed EFS responses, as previously described (Iwatani *et al.*, 2008). However, interaction between endothelium-derived factors and EFS responses are multiple and very complex. Additionally, it should also be considered that specific inhibitors used in the different experiments could also interfere with endothelium-dependent responses, making results more difficult to interpret. Thus, further studies will be needed to analyse endothelium-EFS interactions under the present experimental conditions.

It should be also emphasized that all observed effects of rosuvastatin were not due to direct effects of the statin on the mesenteric vascular wall. In fact, incubation up to three hours with rosuvastatin did not modify the response to EFS in any experimental group. Thus, other mechanisms, such as reduction of non-HDL cholesterol, as well as numerous pleiotropic effects could have contributed to the observed effects of rosuvastatin on mesenteric innervation.

In summary, the present results indicate that neural control of mesenteric vasomotor tone is altered in HFD rats. Both an enhanced adrenergic component and a diminished nitrenergic component

contribute to the observed increase in the vasoconstrictor response to EFS. All of these effects were restored by treatment with rosuvastatin, indicating unknown mechanisms of statins in the neural regulation of vascular tone.

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Conflicts of interest

None to declare.

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