Neurogenic nitric oxide release increases in mesenteric arteries from ouabain hypertensive rats

Fabiano E. Xavier[b,d], Mercedes Salaices[b], Iván Márquez-Rodas[a], María J. Alonso[b], Luciana V. Rossoni[c], Dalton V. Vassallo[d] and Gloria Balfagón[a]

Objectives **We investigated whether chronic ouabain treatment changes the vasoconstrictor responses induced by electrical field stimulation (EFS) in endothelium-denuded rat superior mesenteric arteries and a possible role of neuronal nitric oxide (NO).**

Method **Mesenteric arteries from untreated and ouabain-treated rats (≃8.0 μg/kg per day, for 5 weeks) were used in this study. Vascular reactivity was analyzed by isometric tension recording. Expression of the neuronal NO synthase isoform was analyzed by Western blot. Noradrenaline release was evaluated in segments incubated with [^{3}H]noradrenaline.**

Results **Systolic (SBP) and diastolic (DBP) blood pressure were higher in ouabain-treated rats than in untreated rats (SBP, untreated: 120 ± 3.5 mmHg versus ouabain-treated: 150 ± 4.7 mmHg, $P < 0.01$; DBP, untreated: 87 ± 3.0 mmHg versus ouabain-treated: 114 ± 2.6 mmHg, $P < 0.001$). EFS-induced vasoconstrictions were smaller in arteries from ouabain-treated rats than in those from untreated animals, while the EFS-induced [^{3}H]noradrenaline release and the vasoconstriction induced by exogenous noradrenaline (1 nmol/l–10 μmol/l) remained unmodified. The non-selective NO synthase (NOS) inhibitor, N^G-nitro-L-arginine methyl ester (100 μmol/l), increased the EFS-induced vasoconstriction in mesenteric arteries from both groups, although the effect was more pronounced in segments from ouabain-treated rats. The selective neuronal NOS inhibitor, 7-nitroindazole (7-NI; 100 μmol/l) increased EFS-induced contraction only in segments from ouabain-treated rats. Neuronal NOS expression was greater in the mesenteric arteries from ouabain-treated rats than in those from untreated animals. Sodium nitroprusside (0.1 nmol/l–10 μmol/l) induced a similar vasodilatation in segments from both groups.**

Conclusions **These results suggest that chronic ouabain treatment is accompanied by an increase in neuronal NO release that reduces EFS-induced vasoconstriction.**
J Hypertens **22:949–957**

Journal of Hypertension 2004, **22**:949–957

Keywords: ouabain, hypertension, neuronal nitric oxide, electrical field stimulation, Na^+-pump

Departamentos de [a]Fisiología and [b]Farmacología y Terapéutica, Facultad de Medicina, Universidad Autónoma, Madrid, Spain, [c]Departamento de Fisiologia e Biofísica-ICB, Universidade de São Paulo, SP, Brazil and [d]Pós-Graduação em Ciências Fisiológicas-UFES, Vitória, ES, Brazil.

Sponsorship: This work was supported by grants from Comisión Interministerial de Ciencia y Tecnología (BFI2001-1324 and DGICYT BX 2000-0153) and BSCH. F.E.X. was supported by CAPES (PDEE-BEX0339/02-4, Brazil) and by FISS (C03/01).

Correspondence and requests for reprints to Dr Gloria Balfagón, Departamento de Fisiología Facultad de Medicina, Universidad Autónoma de Madrid. C/Arzobispo Morcillo 4, 28029, Madrid, Spain.
Tel: +34 1 497 5450; fax: +34 1 497 5353; e-mail: gloria.balfagon@uam.es

Received 11 August 2003 **Revised** 16 December 2003
Accepted 7 January 2004

Introduction

One of the characteristics of hypertension is increased vascular resistance caused by a higher smooth muscle tone. Several mechanisms have been proposed to explain this increment in vascular resistance, including endothelial dysfunction [1], increased sympathetic tone [2], structural changes [3,4] or alterations in Na^+, K^+-ATPase activity [5,6], among others.

It is well established that Na^+, K^+-ATPase participates in the modulation of vascular smooth muscle contractility and blood pressure [7]. Lowering the Na^+-pump activity increases intracellular Na^+ concentration, thus reducing the Na^+/Ca^{2+} exchanging activity and consequently increasing intracellular Ca^{2+} concentration and tension [7]. Thus, changes in the functional activity of the vascular Na^+-pump or its regulation may either contribute to the development of hypertension or be a compensatory mechanism against the elevated blood pressure [5,7]. Elevated plasma levels of the endogenous sodium pump inhibitor ouabain have been found in some experimental models of hypertension [6,8], as well as in essential human hypertension [9]. In addition, several reports have demonstrated that chronic administration of ouabain or its structurally related analogs can induce hypertension [10–17].

0263-6352

DOI: 10.1097/01.hjh.0000098300.36684.b7

The hypertension induced by ouabain seems to be the result of a central mechanism associated with increased sympathetic tone, consequent to activation of the central renin–angiotensin system, and resetting of the baroreceptor reflex [11,18]. However, there is also a compensatory or adaptive peripheral mechanism, which we have demonstrated recently, whereby ouabain-induced hypertension is followed by a reduction in vascular reactivity to phenylephrine in the rat aorta and superior mesenteric artery, and increased activity and expression of Na^+, K^+-ATPase in rat aorta [14,15]. This vascular alteration has been associated with increased endothelium-derived nitric oxide (NO) production, probably mediated by the increase in expression of endothelial (eNOS) and neuronal (nNOS) NO synthase isoforms induced by chronic ouabain administration [15]. Other authors [19] also showed, in aortic rings from chronically ouabain-treated Sprague–Dawley rats, a reduction in the contraction inducd by phenylephrine and no changes in the depolarization induced by KCl. However, in renal arteries from chronically ouabain-treated Sprague–Dawley rats, an increase of K^+- and phenylephrine-induced vasoconstriction was observed [20]. The reason for these differences is not entirely clear, but contributing factors may be differences in the vascular bed studied.

It is already known that the activity of the Na^+-pump affects the release of noradrenaline (NA) from sympathetic nerve endings. Electrical field stimulation (EFS) is widely used to study the influence of neurotransmitters released from nerve endings on vasomotor responses [21]. In rat mesenteric arteries, EFS produces a vasoconstrictor response. This response is a balance between the effects resulting from the release of different neurotransmitters, such as NA from adrenergic, neuronal NO from nitrergic and calcitonin gene-related peptide (CGRP) from sensory innervation [22–24]. In addition, evidence of increased neuronal NO release in mesenteric arteries from hypertensive rats has also been reported [24].

Based on the observations cited above, the goal of the present study was to investigate whether ouabain-induced hypertension was associated with changes in vasoconstrictor responses induced by EFS in rat mesenteric arteries, and the possible role of neuronal NO in these changes.

Materials and methods

Animals

Six-week-old male Wistar rats were obtained from the Animal Quarters of the Facultad de Medicina of the Universidad Autónoma de Madrid. Animals were housed at a constant room temperature, humidity and a 12 h light/dark cycle. Rats had free access to tap water and were fed with standard rat chow *ad libitum*. The entire study was conducted in accordance with current Spanish and European laws (RD 223/88 MAPA and 609/86).

Pellet implantation

Rats were anesthetized with diethyl ether and a small incision was made in the back of the neck to implant a subcutaneous controlled time-release pellet (Innovative Research of America, Sarasota, Florida, USA) containing ouabain (0.5 mg/pellet) or vehicle (placebo), as described previously [10]. These pellets are designed to release approximately 8.0 μg/day of ouabain for 60 days.

Blood pressure and heart rate measurements

Five weeks after implantation of the pellets, the rats were anesthetized with ether and the right carotid artery was cannulated with PE-50 tubing filled with heparinized saline. The catheter was tunneled subcutaneously, exteriorized at the nape, and secured to the skin. On the day of the experiments, 24 h later, blood pressure was measured in conscious rats by connecting the arterial catheter to a pressure transducer attached to a data acquisition system (Uni Graph 50; Letica, Barcelona, Spain). Heart rate was determined from the intra-beat intervals of arterial pulses during the experiment.

Tissue preparation

The rats were anesthetized with ether and killed by exsanguination. The superior mesenteric arteries were carefully dissected out, cleaned of connective tissue and placed in Krebs–Henseleit solution (KHS) at 4°C. For experiments of vascular contractility, the arteries were divided into segments of 4 mm length (wet weight: untreated, 4.8 ± 0.32 mg; ouabain-treated, 4.74 ± 0.53 mg; $P > 0.05$). For analysis of nNOS expression, arteries were frozen rapidly in liquid nitrogen and kept at −70°C until the day of analysis.

Contractile responses

Each arterial segment was mounted between parallel wires (75 μm in diameter) in tissue baths at 37°C containing KHS gassed with 95% O_2 and 5% CO_2 to maintain the pH at 7.4. One wire was fixed to the organ bath wall, and the other was connected vertically to the strain gauge for isometric tension recording, according to the method described by Nielsen and Owman [25]. Isometric tension was recorded using an isometric force displacement transducer (Grass FT03C; Massachusetts, USA) connected to a polygraph (Grass Model 7D). For EFS experiments, the segments were mounted between two platinum electrodes, 0.5 cm apart, connected to a stimulator (Cibertec Model CS9; Madrid, Spain), modified to supply adequate current strength. The segments from untreated and ouabain-treated rats were stretched to an optimal resting tension of 0.5 g, which

was re-adjusted every 15 min during a 90-min equilibration period before drug administration. Next, the vessels were exposed to 75 mmol/l K^+ to check their functional integrity. All experiments were performed in endothelium-denuded segments. Endothelium was removed by gently rubbing the intimal surface with a stainless-steel rod. The effectiveness of endothelium removal was confirmed by the absence of the relaxation induced by acetylcholine (ACh, 10 μmol/l) in segments precontracted with 1 μmol/l NA.

Frequency–response curves to EFS or concentration–response curves to NA (1 nmol/l–10 μmol/l) were performed. The parameters used for EFS were 200 mA/0.3 ms/1–8 Hz for 30 s, and a 1-min interval between stimuli, the time required to recover basal tone. A washout period of at least 1 h between consecutive curves was necessary to avoid desensitization. Two successive frequency–response or NA concentration–response curves, separated by 1 h intervals, induced similar contractile responses.

To determine the participation of nitrergic innervation in EFS-induced responses, 100 μmol/l N^G-nitro-L-arginine methyl ester (L-NAME, a non-selective NOS inhibitor) or 100 μmol/l 7-nitroindazole (7-NI, an nNOS inhibitor) was added to the bath 40 min before recording the second frequency–response curve. The effect of L-NAME or 7-NI on the concentration–response curve to NA was also evaluated.

The ability of sodium nitroprusside (SNP, 0.1 nmol/l–10 μmol/l) to induce relaxation in arteries from untreated and ouabain-treated hypertensive rats was assessed in NA (1 μmol/l) precontracted segments.

Tritium overflow

Rat mesenteric artery segments of 4 mm length, from untreated and ouabain-treated rats, were set up in a nylon net and immersed for 90 min in 1 ml of oxygenated KHS at 37°C containing (+)-[^{3}H]NA (0.5 μmol/l, 5 μCi/ml, specific activity 12.8 Ci/mmol). Afterwards, the arteries were transferred to a superfusion chamber with two parallel platinum electrodes, 0.5 cm apart, connected to a stimulator (Cibertec model CS9, modified to supply the adequate current strength) for EFS. The arteries were superfused at a rate of 2 ml/min with oxygenated KHS at 37°C for 60 min, during which the steady-state level of basal tritium efflux was reached. Then, two electrical stimulation periods of 60 s (200 mA, 0.3 ms, 4 Hz; S_1 and S_2) were applied to the arteries at a 60-min interval, and the superfusate was collected in vials (10 in total) at 30-s intervals. These vials were distributed in the following manner: three before stimulation, to determine the basal level of tritium efflux, two during and five after the stimulation; these five vials were enough to recover a basal level of tritium efflux. OptiPhase HiSafe solution (Fisons Chemicals, Loughborough, Leicestershire, UK) was added to the vials and the radioactivity measured in a scintillation counter (Beckman LS 5000 TD; Beckman Instruments, Fullerton, California, USA). 7-NI (100 μmol/l) administered 30 min before S_2 was used to assess if this drug interferes with tritium overflow.

Western blot analysis of nNOS protein expression

Segments of superior mesenteric arteries were homogenized in ice-cold Tris–EDTA buffer (50 mmol/l Tris, 1.0 mmol/l EDTA, pH = 7.4). Homogenates (50 μg protein per lane) and pre-stained molecular weight sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) standards (Bio-Rad, Hercules, California, USA) were separated electrophoretically on 7.5% SDS-PAGE and then transferred to polyvinyl difluoride membranes overnight at 4°C in the Mini Trans-Blot Cell system (Bio-Rad) containing 25 mmol/l Tris, 190 mmol/l glycine, 20% methanol, and 0.05% SDS. Rat pituitary was used for the nNOS-positive control. The membrane was then blocked for 60 min at room temperature in Tris-buffered solution (10 mmol/l Tris, 100 mmol/l NaCl and 0.1% Tween 20) with 5% powdered non-fat milk. Next, the membrane was incubated for 1 h at room temperature with mouse monoclonal antibody for nNOS (1 : 2500), purchased from Transduction Laboratories (Lexington, UK). After washing, the membrane was incubated with a 1 : 2000 dilution of antimouse IgG antibody conjugated to horseradish peroxidase (Transduction Laboratories). The membrane was thoroughly washed, the immunocomplexes were detected with an enhanced horseradish peroxidase/luminol chemiluminescence system (ECL Plus; Amersham International, Little Chalfont, UK) and then subjected to imaging (Hyperfilm ECL; Amersham International). Signals on the immunoblot were quantified using a National Institutes of Health Image V1.56 computer program. The same membrane was used to determine α-actin expression, by means of a monoclonal antibody anti-α-actin (1 : 300 000 dilution, Boehringer Mannheim, Mannheim, Germany) and the content of the latter was used to correct nNOS expression in each sample.

Solutions, drugs and statistical analysis

The composition of KHS was as follows (mmol/l): NaCl 115, $CaCl_2$ 2.5, KCl 4.6, H_2PO_4 1.2, $MgSO_4 \times 7H_2O$ 1.2, $NaHCO_3$ 25, glucose 11.1 and Na_2 EDTA 0.03. Drugs used were NA, ACh, SNP, L-NAME and 7-NI, (Sigma, St. Louis, Missouri, USA). Stock solutions (10 mmol/l) of drugs were made in distilled water, except for NA, which was dissolved in a NaCl (0.9%)–ascorbic acid (0.01% w/v) solution, and 7-NI, which was dissolved in a water–ethanol solution (50% w/v). These solutions were kept at −20°C and appropriate dilutions were made on the day of the experiment.

Contractile responses are expressed as a percentage of the maximum response produced by 75 mmol/l KCl. Relaxation caused by SNP was expressed as a percentage of the initial contraction induced by 1 μmol/l NA. In order to compare the magnitude of effects of 7-NI on the responses to NA in segments from untreated or ouabain hypertensive rats, some results were expressed as 'differences' of area under the concentration–response curves (dAUC) in control and experimental situations. AUC were calculated from the individual concentration–response curves and the differences were expressed as a percentage of the difference to the AUC of the corresponding control situation.

The EFS-induced tritium overflow was calculated by subtracting the basal tritium release from that evoked by electrical stimulation. Ratios of the net tritium overflow between S_2 and S_1 were calculated to eliminate differences between the arteries. The action of the 7-NI on the evoked overflow was expressed as its effects on this ratio. The amount of radioactivity released is indicated in dpm/mg of tissue.

For nNOS expression, results are expressed as the ratio between signals on the immunoblot corresponding to isoforms of nNOS and α-actin. To compare the results for protein expression within the same experiment and with others, we assigned a value of one to the ratio in arteries from untreated rats and used that value to calculate the relative density of other bands from the same gel.

All values are expressed as means ± SEM. '*n*' represents the number of animals used in each experiment. Results were analyzed using Student's *t*-test, by completely randomized two-way ANOVA for comparison between groups, or by repeated-measure ANOVA to compare treatments in the same group. When ANOVA showed a significant treatment effect, Bonferroni's *post hoc* test was used to compare individual means. Differences were considered statistically significant at $P < 0.05$.

Results

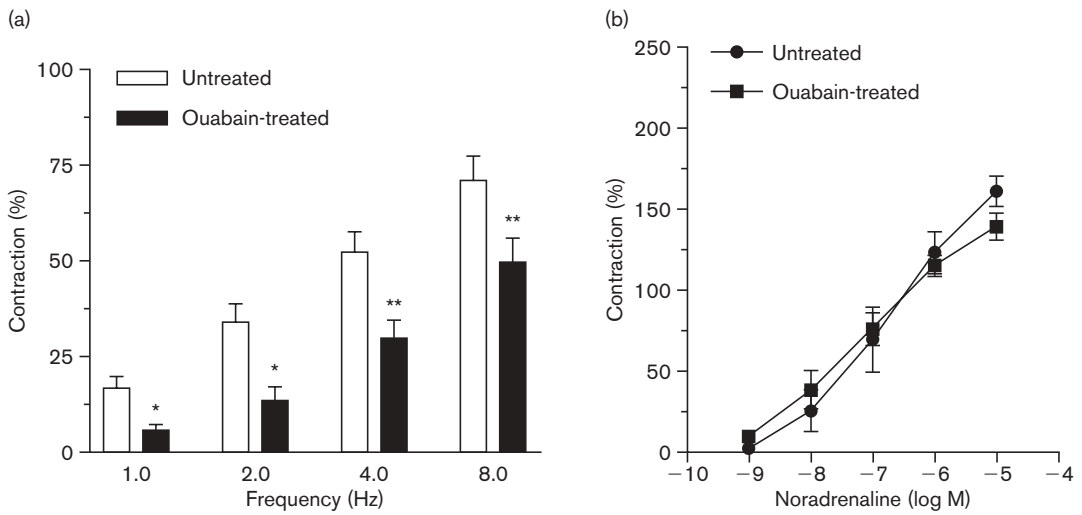
After 5 weeks' treatment the systolic (SBP) and diastolic (DBP) blood pressures were significantly increased in rats treated with ouabain, when compared to untreated rats (SBP: untreated, 120 ± 3.5 mmHg versus ouabain-treated, 150 ± 4.7 mmHg, $P < 0.01$; DBP: untreated, 87 ± 3.0 mmHg versus ouabain-treated, 114 ± 2.6 mmHg, $P < 0.001$). There were no significant differences in heart rate (HR) between the two groups of rats (untreated, 391 ± 13 bpm versus ouabain-treated, 400 ± 18 bpm, $P > 0.05$).

Body weight after 5 weeks of treatment was similar in all groups (untreated, 287 ± 11 g versus ouabain-treated, 293 ± 15 g, $P > 0.05$).

Vascular reactivity

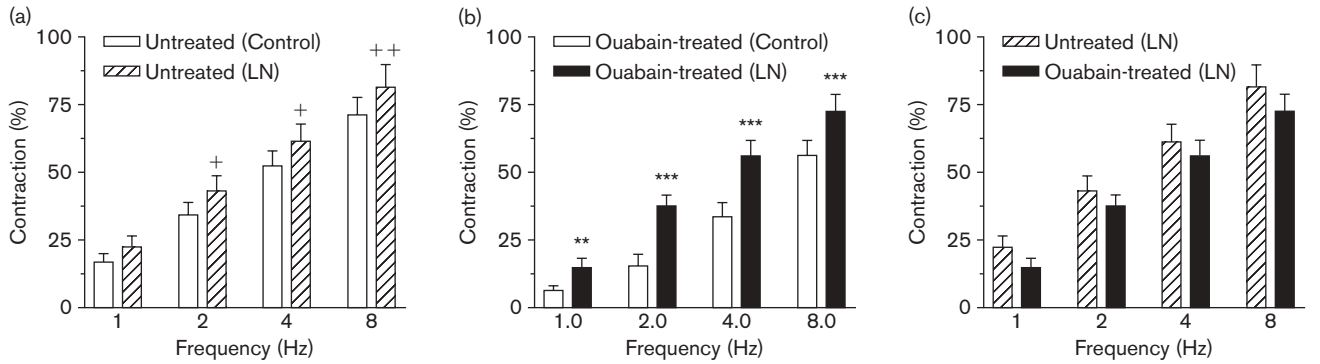
A reduction in vasoconstrictor responses induced by EFS (1, 2, 4 and 8 Hz) was observed in de-endothelialized mesenteric arteries from ouabain-treated rats (Fig. 1a). However, the vascular responses to 75 mmol/l K^+ (untreated, 1241 ± 101 mg versus ouabain-treated, 1257 ± 165 mg, $P > 0.05$) or NA (1 nmol/l–10 μmol/l)

Fig. 1



Frequency-dependent responses (a) and noradrenaline-concentration-dependent (b) contraction in mesenteric artery segments from untreated and ouabain-treated rats. Results (means ± SEM) are expressed as a percentage of the response elicited by 75 mmol/l K^+. $n = 7$ in each group. ANOVA: $^*P < 0.05$, $^{**}P < 0.01$ ouabain-treated versus untreated rats.

Fig. 2



Influence of preincubation with N^G-nitro-L-arginine methyl ester (L-NAME, LN, 100 μmol/l) on the frequency-dependent responses of mesenteric artery segments from untreated (a, $n = 7$) and ouabain-treated rats (b, $n = 7$). (c) compares the electrical field stimulation-induced contraction in the two groups in the presence of L-NAME. Results (means ± SEM) are expressed as a percentage of response elicited by 75 mmol/l K^+. ANOVA: $+P < 0.05$, $++P < 0.01$, untreated (control) versus untreated (LN). $^{**}P < 0.01$, $^{***}P < 0.001$ ouabain-treated (control) versus ouabain-treated (LN).

(Fig. 1b) were not changed by ouabain treatment. Preincubation with the non-selective NOS inhibitor L-NAME (100 μmol/l) significantly increased the EFS-induced contraction in mesenteric arteries from untreated and ouabain-treated rats (Fig. 2a, b). However, this increase was greater in arterial segments from ouabain-treated rats (Table 1). So that, after L-NAME pretreatment, there were no significant differences in the EFS-induced contraction between the two groups studied (Fig. 2c). The L-NAME pretreatment did not modify the vasoconstriction induced by exogenous NA or 75 mmol/l K^+ in either group (results not shown).

Pretreatment with the selective nNOS inhibitor 7-NI (100 μmol/l) increased the EFS-induced contraction significantly only in arterial segments from ouabain-treated rats (Fig. 3a, b). In the presence of 7-NI, the differences in EFS-induced contraction between the two groups studied were abolished (Fig. 3c). Preincubation with 7-NI did not change the K^+-induced contraction in segments from untreated and ouabain-treated rats (results not shown). However, 7-NI significantly reduced the contraction induced by exogenous NA (Fig. 4). This reduction was similar in both groups (dAUC: untreated 37.6 ± 6.68% versus ouabain-treated 37.8 ± 10.1%).

Table 1 Percentage of increment of the electrical field stimulation-induced contraction induced by L-NAME in mesenteric arteries from untreated ($n = 7$) and ouabain-treated rats ($n = 7$)

Frequency (Hz)	Untreated	Ouabain-treated
1	15.3 ± 13%	103.5 ± 34%**
2	20.2 ± 11%	122.7 ± 28%**
4	17.1 ± 6%	80.1 ± 17%**
8	13.6 ± 4%	26.5 ± 5%

L-NAME, N^G-nitro-L-arginine methyl ester. ANOVA: $^{**}P < 0.01$ ouabain-treated versus untreated rats.

In segments from both groups precontracted with 1 μmol/l NA, SNP (0.1 nmol/l–10 μmol/l) induced a similar concentration-dependent relaxation (Fig. 5), which was unmodified by the presence of L-NAME or 7-NI (results not shown).

[^{3}H]NA release experiments

In [^{3}H]NA-preincubated mesenteric arteries from untreated and ouabain-treated rats, two successive electrical pulses induced tritium overflows, which remained unchanged by chronic ouabain treatment: (untreated: $S_1 = 318 \pm 34$ dpm/mg and $S_2 = 277 \pm 46$ dpm/mg versus ouabain-treated: $S_1 = 310 \pm 35$ dpm/mg and $S_2 = 289 \pm 87$ dpm/mg; $P > 0.05$, $n = 6$ in each group). Addition of 100 μmol/l 7-NI, 30 min before S_2, did not modify the basal or stimulated tritium overflow in arteries from both untreated and ouabain-treated rats (untreated: control, $S_2/S_1 = 0.89 \pm 0.08$; 7-NI, $S_2/S_1 = 0.82 \pm 0.11$, $P > 0.05$; ouabain-treated: control, $S_2/S_1 = 0.88 \pm 0.11$; 7-NI, $S_2/S_1 = 0.82 \pm 0.15$, $P > 0.05$).

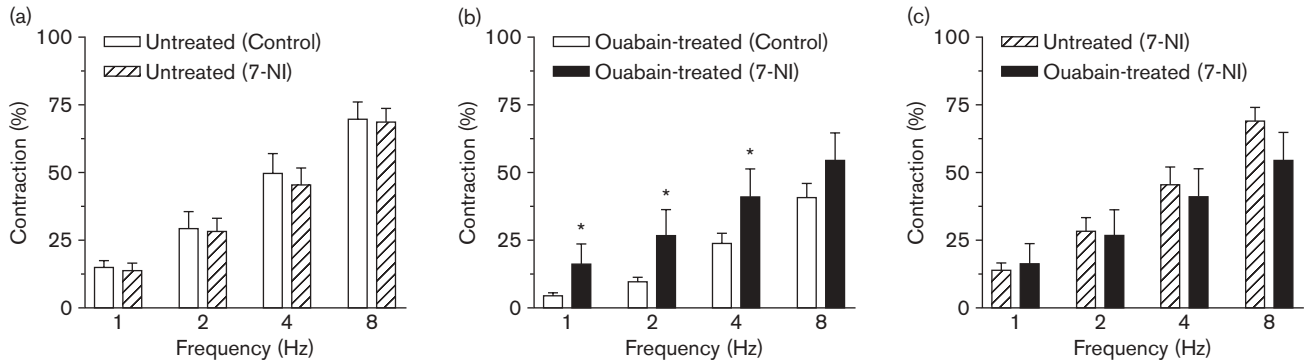
Western blot analysis of nNOS expression

Figure 6 shows that ouabain treatment increased the expression of nNOS in superior mesenteric arteries.

Discussion

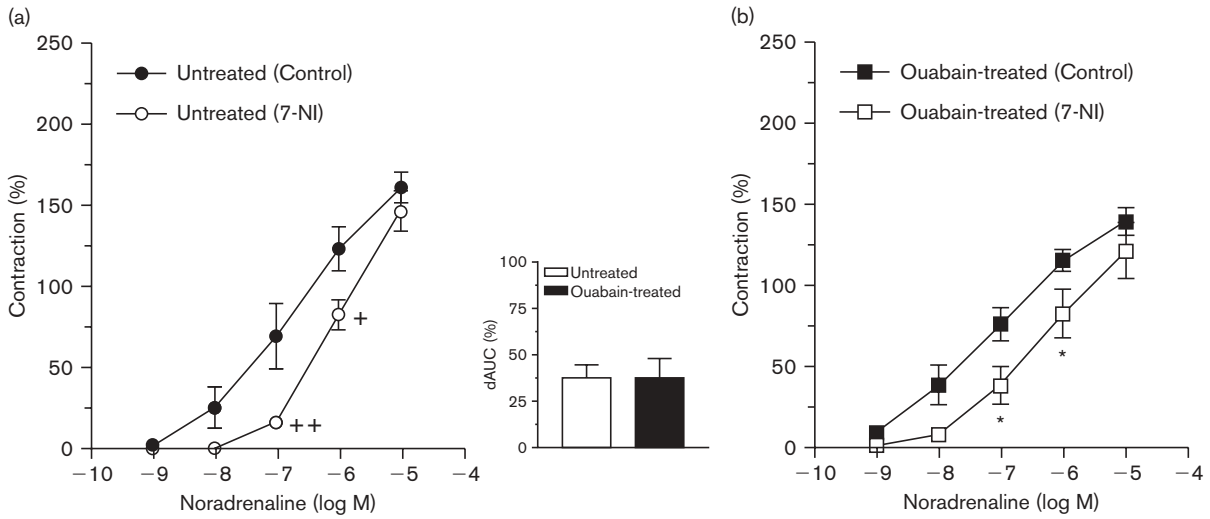
The main and new finding of this work is that hypertension induced by chronic ouabain treatment is accompanied by a reduction in vasoconstrictor response to EFS in de-endothelialized rat mesenteric arteries. This effect seems to be mediated by an increase in neuronal NO release.

Fig. 3



Influence of neuronal nitric oxide synthase (nNOS) inhibition with 7-nitroindazole (7-NI; 100 μmol/l) on the frequency-dependent responses of mesenteric artery segments from untreated (a, $n = 6$) and ouabain-treated rats (b, $n = 7$). (c) The electrical field stimulation-induced contraction in the two groups in the presence of 7-NI. Results (means ± SEM) are expressed as a percentage of response elicited by 75 mmol/l K^+. ANOVA: $\star P < 0.05$ ouabain-treated (control) versus ouabain-treated (7-NI).

Fig. 4

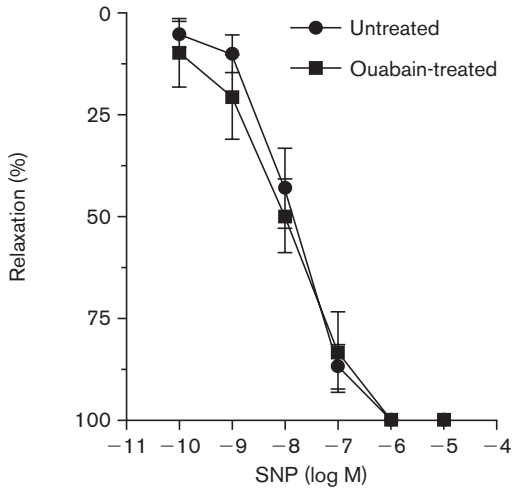


Concentration–response curves to noradrenaline (NA) in endothelium-denuded mesenteric arteries from untreated (a, $n = 6$) and ouabain-treated rats (b, $n = 7$) in the absence and in the presence of 100 μmol/l 7-nitroindazole (7-NI). The insert graph shows differences in area under the concentration–response curve (dAUC) to NA in segments from untreated and ouabain-treated rats in the absence and in the presence of 7-NI. dAUC are expressed as a percentage of the difference of the corresponding AUC for segments in the absence of 7-NI (paired *t*-test, $P > 0.05$). Results (means ± SEM) are expressed as a percentage of response elicited by 75 mmol/l K^+. ANOVA: $+P < 0.05$, $++P < 0.01$ untreated (control) versus untreated (7-NI). $\star P < 0.05$, ouabain-treated (control) versus ouabain-treated (7-NI).

The endogenous digitalis-like compound ouabain is found in the plasma of several mammals and has been associated with the development and/or maintenance of some cardiovascular diseases, including hypertension [6,8,9]. Chronic administration of ouabain leads to increased blood pressure [12,13,17], which has been associated with an increased activity in sympathoexcitatory pathways and a resetting of the baroreceptor reflex [10,11,16]. However, we have recently demonstrated that chronic ouabain treatment reduces the contractile responses to the α-adrenergic agonist phenylephrine in isolated endothelium-intact thoracic aorta and superior mesenteric arteries, a reduction which has been associated with the greater release of endothelial NO [14,15]. Additionally, we have also demonstrated that ouabain-induced hypertension is accompanied by an increase in eNOS and nNOS protein expression in thoracic aorta [15].

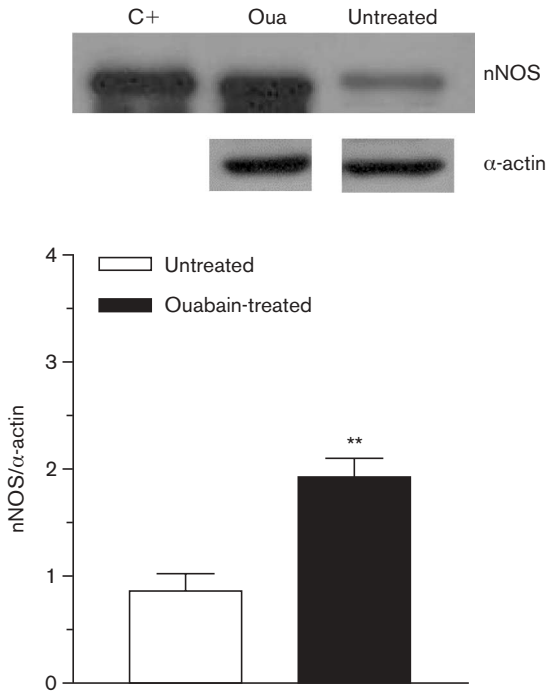
The EFS technique is widely used to study the influence of neurotransmitters released by nervous endings on vasomotor response; NA released from perivascular

Fig. 5



Concentration–response curves to sodium nitroprusside (SNP) in mesenteric arteries from untreated ($n = 7$) and ouabain-treated rats ($n = 7$). Results are expressed as means ± SEM.

Fig. 6



Upper panel: Representative Western blot for neuronal nitric oxide synthase (nNOS) protein expression in superior mesenteric arteries from untreated and ouabain-treated (Oua) rats. The first lane shows the positive control (C+) for nNOS. Lower panel: Densitometric analysis of the Western blot for nNOS protein expression. Results (means ± SEM) are expressed as the ratio between the signal for the nNOS protein and the signal for α-actin. Five to seven arterial samples were used. Unpaired *t*-test: $^{**}P < 0.01$ ouabain-treated versus untreated.

nerve endings is the main neurotransmitter implicated in the vasoconstrictor response induced by EFS in these arteries [26]. However, the entire response depends on the balance between relaxing and contracting agents [26]. Perivascular nitrergic innervation has been described in some vascular beds, including rat mesenteric arteries, as an important source of NO, a substance that induces vasodilatation and participates in the regulation of the vascular tone [26–28]. Since hypertension induced by ouabain seems to be associated with increases in vascular NO production [15] as well as with sympathetic nerve activity [29], we analyzed the possible alterations of EFS-induced responses in mesenteric arteries from ouabain-treated rats and the possible role of neuronal NO production in these alterations.

The contractile responses induced by EFS were reduced in endothelium-denuded segments from ouabain-treated rats, compared to untreated rats. Based on previous results obtained by our group [14] the changes observed in the current work after ouabain treatment do not seem to be due to alterations in the expression of the α_1 or α_2 isoforms of the catalytic subunit of Na^+, K^+-ATPase, or in the activity of this enzyme, because in the mesenteric arteries both parameters remained unmodified by ouabain treatment. On the other hand, this reduction was not due to alterations in the NA release from perivascular nerve endings or its effects on vascular smooth muscle, since both [^{3}H]NA release and NA-induced vasoconstrictor responses remained unmodified after ouabain chronic treatment. To determine whether alterations in NO release could account for the changes in vasoconstrictor responses induced by EFS in segments from ouabain-treated rats, arterial segments from both groups were preincubated with the non-selective NOS inhibitor L-NAME. Inhibition of NOS significantly increased the vasoconstrictor response to EFS in segments from both groups, indicating the participation of non-endothelial NO in the contractile responses induced by EFS. However, the effect of L-NAME was greater in mesenteric arteries from ouabain-treated rats than in those from untreated rats. Since the differences in EFS-induced responses between untreated and ouabain-treated rats were abolished by preincubation with L-NAME and ouabain treatment did not change NA-induced contraction, our results reflect the existence of constitutively available non-endothelial NO that would modulate EFS-induced contractile responses by vascular smooth muscle in segments from ouabain-treated rats. Similar results were observed in mesenteric arteries from spontaneously hypertensive rats (SHR), where the neuronal NO release induced by EFS is also increased [24]. These results lead us to suggest that increased neuronal NO release can be one of the mechanisms of adaptation developed to compensate for the increased total peripheral resistance subsequent to hypertension. However,

the results with respect to the alteration of NO synthesis or breakdown in hypertension are controversial. Thus, evidence of attenuated [30], unaltered [20,31] or enhanced [15,24,32,33] NO synthesis has been demonstrated in several vascular beds from hypertensive animals. In hypertension induced by ouabain, no change in the endothelial NO release induced by ACh in renal, aorta, mesenteric and caudal arteries has been reported [14,20]. However, the role of endothelial NO in the vasoconstrictor responses to phenylephrine were greater in arteries from rats treated with ouabain [15], suggesting that ouabain-induced hypertension is accompanied by increased NO release derived from endothelial NOS.

Our results suggested a role for neuronal NO in EFS-induced responses, and the nNOS inhibitor, 7-NI, was used to confirm this possibility. Although 7-NI has been used widely as a selective nNOS inhibitor [34], it has been suggested that this drug could have the same potency to inhibit eNOS and nNOS activity [34]. In rat superior mesenteric arteries, 100 μmol/l 7-NI reduced the ACh-induced relaxation, but to a lesser extent than that observed with 100 μmol/l L-NAME (unpublished results). Similar results have been found by other investigators in rat aorta [35], indicating that these drugs do not have the same potency to inhibit endothelial and neuronal NO release. On the other hand, in our experiments evaluating the role of NO on EFS-induced vasoconstrictor responses in untreated and ouabain-treated rats, we used de-endothelialized arteries, eliminating the possibility that 7-NI was affecting the NO synthesis induced by eNOS. In addition, Ayajiki *et al.* [36] reported that 100 μmol/l 7-NI completely abolished the neuronal NO-dependent relaxation induced by EFS or nicotine in de-endothelialized monkey cerebral arteries. 7-NI increased the vasoconstriction induced by EFS in segments from ouabain-treated rats but did not modify EFS responses in segments from untreated rats. This effect of 7-NI is not due to alterations of NA release, since this drug did not modify the tritium overflow in segments from both experimental groups. However, 7-NI reduced vasoconstrictor responses to exogenous NA to a similar extent in both groups. These results could help to explain the absence of an increase of EFS-induced contraction in segments from untreated rats, as that observed in L-NAME preincubated arteries, and support the hypothesis that an increased NO release from nNOS could be responsible for the decreased EFS-induced vasoconstriction in mesenteric arteries from ouabain-induced hypertensive rats. Reinforcing this suggestion, nNOS expression was increased in segments from ouabain hypertensive rats. Similarly increases in nNOS expression have been reported in aorta from ouabain-treated rats [15], as well as in resistance mesenteric arteries and carotid arteries from SHR [37,38].

The mechanism involved in the non-specific effects of 7-NI on NA-induced contraction is not known. However, other investigators [39,40] have reported similar results related with an alteration of cellular calcium influx [39]. In the present work, the K^+-induced contraction was not changed by 7-NI treatment, which suggests that the calcium influx through voltage-dependent calcium channels is not inhibited. However, inhibition by 7-NI of the other calcium mobilization pathway activated by NA cannot be ruled out.

The relaxation induced by SNP was similar in NA-precontracted mesenteric arteries from ouabain-treated and untreated rats, and was unaffected by L-NAME or 7-NI. These results rule out the possibility that the greater potentiation induced by L-NAME or 7-NI in segments from ouabain-treated rats could be due to changes in the sensitivity of vascular smooth muscle to NO.

Since EFS can liberate other neurotransmitters as well as NA and NO, it is possible that other neurotransmitters release will also be altered in the mesenteric arteries from ouabain-treated rats. However, these possible alterations do not seem to have functional implications, since after NOS inhibition no differences were detected in EFS-induced responses between the two study groups.

In summary, the results presented here suggest that chronic ouabain treatment reduces vasoconstriction induced by EFS in rat mesenteric arteries. This alteration seems to be a consequence of an increased neuronal NO release, probably associated with increased nNOS protein expression. These neuronal alterations seem to constitute a counter-regulatory mechanism against the elevated blood pressure present in these animals.

Acknowledgements

We are grateful to Dr M.C. Fernandéz-Criado for the care of animals and Miss Rocío Baena Porras and Marta Miguel for their technical assistance.

References

1 Vanhoutte PM. Endothelium and control of vascular function. State of the art lecture. *Hypertension* 1989; **13**:658–667.
2 Abboud FM. The sympathetic system in hypertension: state of the art review. *Hypertension* 1982; **4**:208–225.
3 Intengan HD, Schiffrin EL. Structure and mechanical properties of resistance arteries in hypertension: Role of adhesion molecules and extracellular matrix determinants. *Hypertension* 2000; **36**:312–318.
4 Mulvany MJ. Small artery remodeling in hypertension. *Curr Hypertens Rep* 2002; **4**:49–55.
5 Marín J, Redondo J. Vascular sodium pump: endothelial modulation and alterations in some pathological processes and aging. *Pharmacol Ther* 1999; **84**:249–271.
6 Overbeck HW, Pammani MB, Akera T, Brody TM, Haddy FJ. Depressed function of an ouabain-sensitive sodium–potassium pump in blood vessels from renal hypertensive dogs. *Circ Res* 1976; **38**:II48–52.
7 Blaustein MP. Physiological effects of endogenous ouabain: control of

intracellular Ca^{2+} stores and cell responsiveness. *Am J Physiol* 1993; **264**:C1367–1387.
8 Hamlyn JM, Hamilton BP, Manunta P. Endogenous ouabain, sodium balance and blood pressure: a review and a hypothesis. *J Hypertens* 1996; **14**:151–171
9 Hamlyn JM, Ringel R, Schaeffer J, Levinson PD, Hamilton BP, Kowearski AA, Blaustein MP. A circulating inhibitor of (Na^++K^+)ATPase associated with essential hypertension. *Nature* 1982; **300**:650–652.
10 Huang BS, Huang X, Harmsen E, Leenen FHH. Chronic central versus peripheral ouabain, blood pressure, and sympathetic activity in rats. *Hypertension* 1994; **23**:1087–1090.
11 Huang BS, Leenen FHH. Brain renin–angiotensin system and ouabain-induced sympathetic hyperactivity and hypertension in Wistar rats. *Hypertension* 1999; **34**:107–112.
12 Manunta P, Rogowski AC, Hamilton BP, Hamlyn JM. Ouabain-induced hypertension in the rat: relationships among plasma and tissue ouabain and blood pressure. *J Hypertens* 1994; **12**:549–560.
13 Manunta P, Hamilton BP, Hamlyn JM. Structure–activity relationship for the hypertensinogenic activity of ouabain: role of the sugar and lactone ring. *Hypertension* 2001; **37**:471–477.
14 Rossoni LV, Salaices M, Marín J, Vassallo DV, Alonso MJ. Alterations on vascular reactivity to phenylephrine and Na^+,K^+-ATPase activity and expression in hypertension induced by chronic administration of ouabain. *Br J Pharmacol* 2002; **135**:771–781.
15 Rossoni LV, Salaices M, Miguel M, Briones AM, Barker LA, Vassallo DV, Alonso MJ. Ouabain-induced hypertension is accompanied by increases in endothelial vasodilator factors. *Am J Physiol* 2002; **283**:H2110–2118.
16 Veerasingham SJ, Leenen FHH. Ouabain- and central sodium-induced hypertension depend on the ventral anteroventral third ventricle region. *Am J Physiol* 1999; **276**:H63–70.
17 Yuan CM, Manunta P, Hamlyn JM, Chen S, Bohen E, Yeun J, *et al*. Long-term ouabain administration produces hypertension in rats. *Hypertension* 1993; **22**:178–187.
18 Zhang J, Leenen FHH. AT(1) receptor blockers prevent sympathetic hyperactivity and hypertension by chronic ouabain and hypertonic saline. *Am J Physiol* 2001; **280**:H1318–1323.
19 Cargnelli G, Trevisi L, Debetto P, Luciani S, Bova S. Effect of long-term ouabain treatment on contractile responses of rat aortae. *J Cardiovasc Pharmacol* 2000; **35**:538–542.
20 Kimura K, Manunta P, Hamilton BP, Hamlyn JM. Different effects of in vivo ouabain and digoxin on renal artery function and blood pressure in the rat. *Hypertens Res* 2000; **23**:S67–76.
21 Loesch A. Perivascular nerves and vascular endothelium: recent advances. *Histol Histopathol* 2002; **17**:591–597.
22 Kawasaki H, Takasaki K, Saito A, Goto K. Calcitonin gene-related peptide acts as a novel vasodilator neurotransmitter in mesenteric resistance vessels of the rat. *Nature* 1988; **335**:164–167.
23 Marín J, Balfagón G. Effect of clenbuterol on non-endothelial nitric oxide release in rat mesenteric arteries and the involvement of beta-adrenoceptors. *Br J Pharmacol* 1998; **124**:473–478.
24 Marín J, Ferrer M, Balfagón G. Role of protein kinase C in electrical-stimulation-induced neuronal nitric oxide release in mesenteric arteries from hypertensive rats. *Clin Sci* 2000; **99**:277–283.
25 Nielsen KC, Owman C. Contractile response and amine receptor mechanisms in isolated middle cerebral artery of the cat. *Brain Res* 1971; **27**:25–32.
26 Lee TJ. Nitric oxide and the cerebral vascular function. *J Biomed Sci* 2000; **7**:16–26.
27 Moll-Kaufmann C, Sumanovski LT, Sieber CC. Neurally-mediated vasodilatation in normal and portal hypertensive rats: role of nitric oxide and calcitonin gene-related peptide. *J Hepatol* 1998; **28**:1031–1036.
28 Toda N, Okamura T. Neurogenic nitric oxide (NO) in the regulation of cerebroarterial tone. *J Chem Neuroanat* 1996; **10**:259–265.
29 Aileru AA, De Albuquerque A, Hamlyn JM, Manunta P, Shah JR, Hamilton MJ, Weinreich D. Synaptic plasticity in sympathetic ganglia from acquired and inherited forms of ouabain-dependent hypertension. *Am J Physiol* 2001; **281**:R635–644.
30 Chou TC, Yen MH, Li CY, Ding YA. Alterations of nitric oxide synthase expression with aging and hypertension in rats. *Hypertension* 1998; **31**:643–648.
31 Briones AM, Alonso MJ, Hernanz R, Miguel M, Salaices M. Alterations of the nitric oxide pathway in cerebral arteries from spontaneously hypertensive rats. *J Cardiovasc Pharmacol* 2002; **39**:378–388.
32 Dubey RK, Boegehold MA, Gillespie DG, Rosselli M. Increased nitric oxide activity in early renovascular hypertension. *Am J Physiol* 1996; **270**:R118–124.
33 Maffei A, Poulet R, Vecchione C, Colella S, Fratta L, Frati G, *et al*. Increased basal nitric oxide release despite enhanced free radical production in hypertension. *J Hypertens* 2002; **20**:1135–1142.
34 Moore PK, Handy RCL. Selective inhibitors of neuronal nitric oxide synthase – is no NOS really good NOS for the nervous system? *Trends Pharmacol Sci* 1997; **18**:204–211.
35 Ralevic V. Endothelial nitric oxide modulates perivascular sensory neurotransmission in the rat isolated mesenteric arterial bed. *Br J Pharmacol* 2002; **137**:19–28.
36 Ayajiki K, Fujioka H, Okamura T, Toda N. Relatively selective neuronal nitric oxide synthase inhibition by 7-nitroindazole in monkey isolated cerebral arteries. *Eur J Pharmacol* 2001; **423**:179–183.
37 Boulanger CM, Heymes C, Benessiano J, Geske RS, Levy BI, Vanhoutte PM. Neuronal nitric oxide synthase is expressed in rat vascular smooth muscle cells: activation by angiotensin II in hypertension. *Circ Res* 1998; **83**:1271–1278.
38 Briones AM, Alonso MJ, Marin J, Balfagon G, Salaices M. Influence of hypertension on nitric oxide synthase expression and vascular effects of lipopolysaccharide in rat mesenteric arteries. *Br J Pharmacol* 2000; **131**:185–194.
39 Allawi HS, Wallace P, Pitcher A, Galfen Z, Bland-Ward PA, Moore PK. Effect of 7-nitro indazole on neurotransmission in the rat vas deferens: mechanisms unrelated to inhibition of nitric oxide synthase. *Br J Pharmacol* 1994; **113**:282–288.
40 Ollerstam A, Salomonsson M, Persson AE. Reduced rat renal vascular response to angiotensin II after chronic inhibition of nNOS. *Acta Physiol Scand* 2002; **176**:245–252.