Ouabain-induced hypertension alters the participation of endothelial factors in α-adrenergic responses differently in rat resistance and conductance mesenteric arteries

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1   This study compares the role of endothelial factors in α-adrenoceptor contractile responses in mesenteric resistance (MRA) and superior (SMA) mesenteric arteries from ouabain-treated ($8.0\,\mu g\,day^{-1}$, 5 weeks) and untreated rats. The role of the renin–angiotensin system was also evaluated.

2   Ouabain treatment increased systolic blood pressure. In addition, ouabain reduced the phenylephrine response in SMA but did not alter noradrenaline responses in MRA.

3   Endothelium removal or the nitric oxide synthase (NOS) inhibitor (L-NAME, $100\,\mu M$) increased the responses to α-adrenergic agonists in both vessels. After ouabain treatment, both endothelial modulation and the L-NAME effect were increased in SMA, while only the L-NAME effect was increased in MRA. Endothelial NOS expression remained unaltered after ouabain treatment.

4   Indomethacin ($10\,\mu M$) similarly reduced the noradrenaline contraction in MRA from both groups; in contrast, in SMA, indomethacin only reduced phenylephrine-induced contractions in segments from untreated rats. Co-incubation of L-NAME and indomethacin leftward shifted the concentration–response curves for noradrenaline more in MRA from ouabain-treated rats; tetraethylammonium ($2\,mM$) shifted the noradrenaline curves further leftward only in MRA from untreated rats.

5   Losartan treatment prevents the development of hypertension but not all vascular changes observed after ouabain treatment.

6   In conclusion, a rise in endothelial NO and impaired prostanoid participation might explain the reduction in phenylephrine-induced contraction in SMA after ouabain treatment. An increase in the modulatory effect of endothelial NO and impairment of endothelium-dependent hyperpolarizing factor effect might explain why the ouabain treatment had no effect on noradrenaline responses in MRA.
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Abbreviations: ACh, acetylcholine; COX, cyclooxygenase; dAUC, difference of area under the concentration–response curves; EDHF, endothelium-derived hyperpolarizing factor; EDTA, ethylenediamine-tetraacetic acid; E_{max}, maximum response; eNOS, endothelial nitric oxide synthase; KHS, Krebs–Henseleit solution; MRA, mesenteric resistance arteries; L-NAME, N-nitro-L-arginine methyl ester; NO, nitric oxide; pD_2, negative logarithm of concentrations producing 50% of maximum response; SBP, systolic blood pressure; SMA, superior mesenteric arteries; TEA, tetraethylammonium

Introduction

The role of an endogenous ouabain-like compound in the development and/or maintenance of hypertension has been suggested by findings that show that its plasma levels are elevated in some forms of hypertension. In addition, exogenous ouabain, as well as its structurally related analogs, induces hypertension when chronically administered to normotensive rats (Hamlyn *et al.*, 1982; 1996; Huang *et al.*, 1994; Manunta *et al.*, 1994; 2001; Rossoni *et al.*, 2002a, b; Di Filippo *et al.*, 2003). This hypertension seems to depend, at least in part, on

central mechanisms associated with increased sympathetic tone, subsequent to the activation of cerebral renin–angiotensin (Huang & Leenen, 1999; Zhang & Leenen, 2001) and endothelin (Di Filippo *et al.*, 2003) systems.

Additional peripheral mechanisms might occur in the hypertension induced by chronic treatment with ouabain. Previously, we have demonstrated that this hypertensive model is accompanied by a reduction in vascular reactivity to phenylephrine and by increased activity and expression of Na^+, K^+-ATPase in rat aortae (Rossoni *et al.*, 2002a, b). These alterations are associated with increased endothelium-derived nitric oxide (NO) production and increased expression

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of the endothelial (eNOS) and neuronal isoforms of NO synthase (Rossoni *et al*., 2002b; Xavier *et al*., 2004). Other authors have also shown in aortic rings from ouabain-treated rats a reduction in the contraction induced by phenylephrine in the absence of a development of hypertension (Cargnelli *et al*., 2000). However, in isolated renal arterial preparations, treatment with ouabain was accompanied by increased K^+- and phenylephrine-induced vasoconstriction (Kimura *et al*., 2000). Collectively, these observations suggest that different vascular beds are differently affected by ouabain treatment.

All the studies mentioned above used conductance arteries. However, at present, there are no published studies on the effects of chronic treatment with ouabain on resistance arteries, despite the fact that resistance arteries have a much greater role in the regulation of the total peripheral vascular resistance and blood pressure than do conductance vessels. The purpose of this study was to compare the effect of hypertension induced by ouabain treatment on the endothelial modulation of vascular responses induced by α-adrenoceptor activation in resistance and conductance arteries. For this purpose, the main and the fourth branch of superior mesenteric arteries were used. We also studied whether antihypertensive treatment with an AT_1-angiotensin receptor antagonist (losartan), which has been effectively demonstrated to block the hypertensinogenic effect of ouabain (Zhang & Leenen, 2001), would prevent the possible vascular alterations induced by chronic ouabain treatment.

Methods

Male Wistar rats, 6-week-old, were obtained from colonies maintained at the Animal Quarters of the Facultad de Medicina of the Universidad Autónoma de Madrid. All experiments comply with the current Spanish and European laws (RD 223/88 MAPA and 609/86).

Rats were divided into four treatment groups: untreated, ouabain-treated ($\approx 8.0\,\mu g\,day^{-1}$), losartan-treated ($15\,mg\,kg^{-1}\,day^{-1}$, v.o.) and ouabain plus losartan-treated. Controlled time-release pellets (Innovative Research of America) containing ouabain (0.5 mg) or vehicle (placebo) were subcutaneously implanted, as described previously (Huang *et al*., 1994). Losartan, administered from the time the pellets were implanted, was dissolved in tap water at a final concentration, calculated as a function of body weight and the volume of water consumed. Systolic blood pressure (SBP) was measured by the tail-cuff method before the beginning of treatment and every week during treatment until week 5.

Vascular reactivity measurements

After 5-weeks' treatment, rats were decapitated and mesenteric arteries removed. Segments of superior mesenteric arteries 4 mm in length were set up in gassed (95% O_2 and 5% CO_2) Krebs–Henseleit solution (KHS), at 37°C, under a tension of 0.5 g. Isometric tension was recorded using an isometric force displacement transducer (Grass FT03C, MA, U.S.A.) connected to an acquisition system (MacLab/41 ADInstruments Pty Ltd, Castle Hill, Australia). Segments of fourth-order mesenteric resistance arteries, 2 mm in length, were mounted in a small-vessel dual-chamber myograph for measurement of

isometric tension according to the method described by Mulvany & Halpern (1977).

Experimental protocols

After a 45 min equilibration period, mesenteric resistance and superior mesenteric arteries were exposed to 120 and 75 mM KCl, respectively, to check their functional integrity. Then, concentration–response curves to acetylcholine (ACh, 0.01 nM–30 μM) were performed in mesenteric resistance and superior mesenteric arteries previously contracted with noradrenaline or phenylephrine, respectively, at a concentration that produced approximately 50% of the contraction induced by KCl in each case.

After a washout period of 60 min, concentration–response curves to phenylephrine (1 nM–10 μM) were constructed in superior mesenteric arteries. In resistance arteries, noradrenaline (10 nM–30 μM) was used to study the vasoconstriction induced by α-adrenoceptor activation because noradrenaline produced responses that were more stable than those produced by phenylephrine; the tonic phase of the contractions induced by phenylephrine was not sustained and this precluded the generation of cumulative concentration–response curves. In some experiments, the role of the endothelium on the contractile responses was studied by removing this vascular component. In other experiments, the effects of the NOS inhibitor *N*-nitro-L-arginine methyl ester (L-NAME, 100 μM) and the cyclooxygenase (COX) inhibitor indomethacin (10 μM) on the noradrenaline- or phenylephrine-induced response were investigated. These drugs were added 30 min before the concentration–response curve.

In another set of experiments, the role of endothelium-derived hyperpolarizing factor (EDHF) on noradrenaline-induced contraction was assessed in mesenteric resistance arteries from the experimental groups studied. For this, three consecutive concentration–response curves to noradrenaline were performed at intervals of 1 h; L-NAME (100 μM) plus indomethacin (10 μM) and L-NAME plus indomethacin plus tetraethylammonium (TEA, 2 mM) were added 30 min before the second and the third concentration–response curves, respectively. No differences between the three consecutive concentration–response curves to noradrenaline were observed in control experiments (results not shown).

Western blot analysis of eNOS protein expression

Proteins from homogenized mesenteric arteries (30 μg per lane) were separated by 7.5% sodium lauryl sulphate-polyacrylamide gel and then transferred to polyvinyl difluoride membranes overnight. Next, the membranes were incubated for 1 h with mouse monoclonal antibody for eNOS (1 : 2500, Transduction Laboratories, Lexington, U.K.) and, after washing, incubated with antimouse IgG antibody conjugated to horseradish peroxidase (1 : 2000, Transduction Laboratories). The immunocomplexes were detected using an enhanced horseradish peroxidase/luminol chemiluminescence system (ECL Plus, Amersham International plc, Little Chalfont, U.K.) and subjected to autoradiography (Hyperfilm ECL, Amersham International plc). Signals on the immunoblot were quantified using a computer program (NIH Image V1.56). The same membranes were used to determine α-actin expression using a mouse monoclonal antibody (1 : 3,000,000, Boehringer

Mannheim, Mannheim, Germany). Homogenates from human endothelial cells were used for eNOS-positive control.

Solutions, drugs and statistical analysis

The composition of KHS was as follows (mM): NaCl 115, $CaCl_2$ 2.5, KCl 4.6, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.2, $NaHCO_3$ 25, glucose 11.1 and Na_2EDTA 0.03. Losartan was a gift of Merck Sharp & Dohme (Spain). Drugs used were phenylephrine hydrochloride, noradrenaline hydrochloride, ACh chloride, L-NAME dihydrochloride, indomethacin and TEA (Sigma, St Louis, MO, U.S.A.). Stock solutions (10 mM) of drugs were made in a distilled water, except for noradrenaline, which was dissolved in a NaCl (0.9%)-ascorbic acid (0.01% w v^{-1}) solution, and indomethacin, which was dissolved in 1.5 mM $NaHCO_3$. 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 KCl. The maximum effect (E_{max}) and the concentration of agonist that produced half of E_{max} ($\log EC_{50}$) were calculated using nonlinear regression analysis (GraphPad Prism Software, San Diego, CA, U.S.A.). The sensitivity of the agonists is expressed as pD_2 ($-\log EC_{50}$). Some results were expressed as 'differences' of area under the concentration–response curves (dAUC) to phenylephrine or noradrenaline in control and experimental situations. AUC were calculated from the individual concentration–response curves plot (GraphPad Prism Software, San Diego, CA, U.S.A.) and the differences were expressed as a percentage of the difference to AUC of the corresponding control situation. Results of eNOS expression are expressed as the ratio between optical density for NOS and for α-actin.

All values are expressed as means±s.e.m. of 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 two-way ANOVA to compare treatments within 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

Ouabain treatment induced a progressive increase in SBP from the first week ($P < 0.05$). In both ouabain-treated and untreated rats, treatment with losartan reduced the SBP to a similar level (Figure 1). The body weight was similar in all experimental groups studied (untreated: 324 ± 12; ouabain-treated: 335 ± 15; losartan-treated: 343 ± 29; ouabain + losartan-treated: 326 ± 28 g, $P > 0.05$).

KCl (120 mM in mesenteric resistance arteries and 75 mM in superior mesenteric arteries) evoked contractions in vessels from ouabain-treated rats that were similar to those obtained in vessels from untreated rats for both the mesenteric resistance (untreated: 9.5 ± 0.8 vs ouabain-treated: 9.8 ± 0.8 mN, $P > 0.05$) and superior mesenteric (untreated: 12.2 ± 1.0 vs ouabain-treated: 13.3 ± 0.6 mN, $P > 0.05$) arteries. Treatment with losartan did not change KCl response (results not shown) in either type of arteries from ouabain-treated and untreated rats. The endothelium-dependent relaxation to ACh (0.01 nM–30 μM) also remained unmodified in all study groups (results not shown).
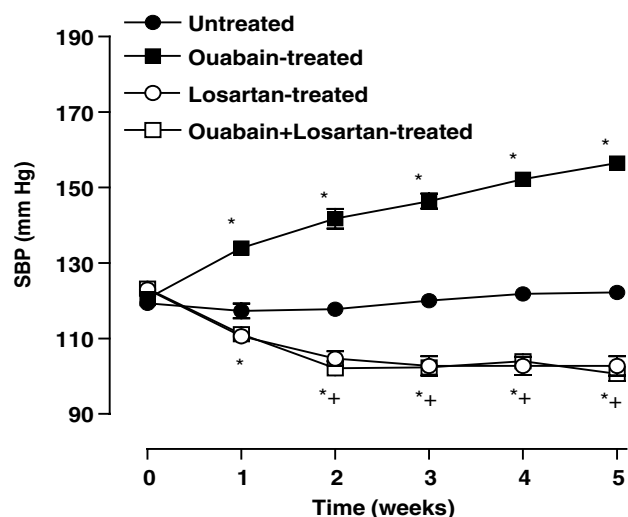

Figure 1 SBP for Wistar rats, which received vehicle (untreated $N = 17$), ouabain ($N = 23$), losartan ($N = 7$) or ouabain plus losartan ($N = 8$). Results are expressed as means ± s.e.m. for the number of animals used. *$P < 0.05$ vs untreated rats; $^{+}P < 0.05$ vs untreated/ ouabain-treated rats.

Contractile responses mediated by α-receptor activation in ouabain-treated and untreated rats

Contractile responses to noradrenaline were similar in mesenteric resistance arteries from ouabain-treated and untreated rats (Figure 2 and Table 1) and remained unmodified after losartan treatment (Figure 2 and Table 2). However, in superior mesenteric arteries, the contractile responses produced by phenylephrine were smaller in ouabain-treated than in untreated rats (Figure 2 and Table 1) and remained smaller in segments from rats that received ouabain plus losartan (Figure 2 and Table 2).

Effect of endothelium removal on α-adrenergic responses

In both ouabain-treated and untreated rats, endothelium removal increased the potency of the agonists in both types of artery, while the maximum response was only increased for phenylephrine in superior mesenteric arteries (Figure 3 and Table 1). The effect of endothelium removal was larger in superior mesenteric than in mesenteric resistance arteries (see dAUC graph in Figure 3).

In mesenteric resistance arteries, the potentiation of noradrenaline-induced contraction by endothelium removal was similar in both experimental groups. In contrast, in superior mesenteric arteries, the potentiation of the phenylephrine response was greater in preparations from ouabain-treated than those from untreated rats (see dAUC graph in Figure 3).

Treatment with losartan did not alter the effect induced by endothelium removal on α-adrenergic responses in both arteries from ouabain-treated and untreated rats (Table 2).

Effect of NOS inhibition on α-adrenergic responses

To assess the contribution of endothelium-derived NO to the alpha-adrenergic responses, segments were incubated with the
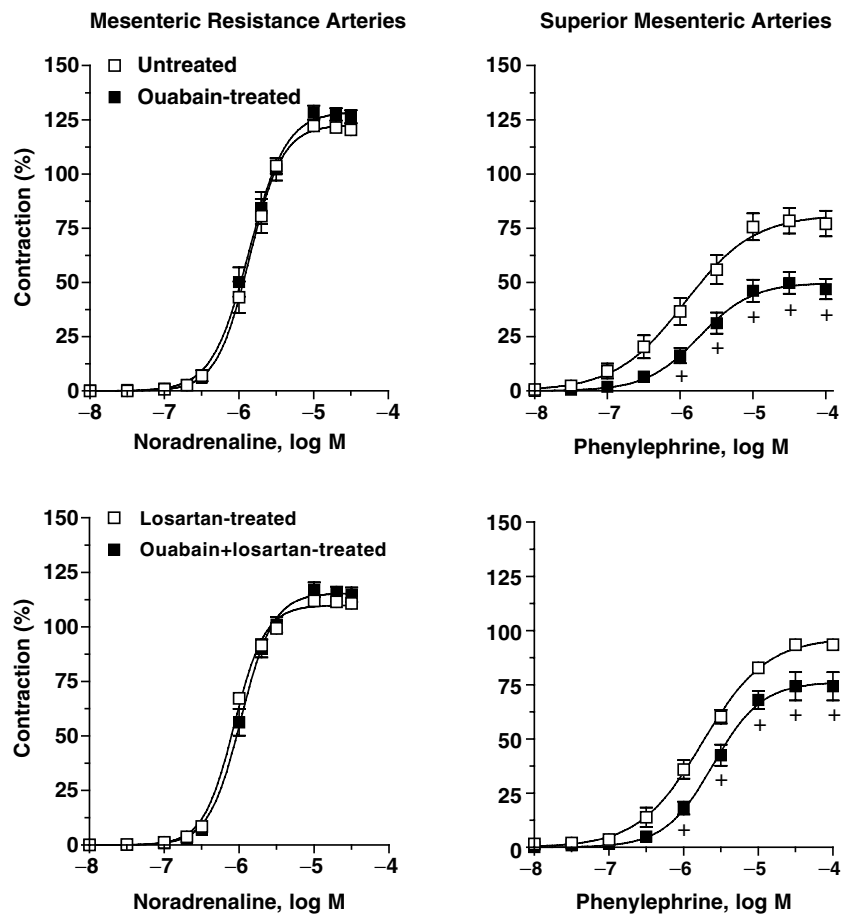
Figure 2 Contractile responses to α-adrenoceptor activation in superior mesenteric arteries and mesenteric resistance arteries from untreated ($N = 7, 9$) and ouabain-treated ($N = 7, 9$) or losartan-treated ($N = 7, 7$) and ouabain plus losartan-treated ($N = 8, 8$) rats. Results (means ± s.e.m.) are expressed as a percentage of response elicited by KCl. ANOVA (two-way): $^{+}P < 0.05$.

Table 1 Effect of endothelium denudation (E−), L-NAME and indomethacin treatment on E_{max} and pD_2 to noradrenaline in MRA and to phenylephrine in SMA from rats subcutaneously receiving vehicle (untreated) and ouabain (Oua) for 5 weeks

	MRA		*SMA*	
	E_{max}	pD_2	E_{max}	pD_2
Control				
Untreated	121 ± 2	5.93 ± 0.04	80 ± 6	6.00 ± 0.09
Oua-treated	128 ± 3	5.98 ± 0.03	52 ± 5^{+}	5.67 ± 0.10
E−				
Untreated	122 ± 3	6.58 ± 0.08*	103 ± 5*	6.87 ± 0.09*
Oua-treated	129 ± 7	6.42 ± 0.08*	100 ± 4*	6.66 ± 0.09*
L-NAME				
Untreated	127 ± 5	6.17 ± 0.05*	135 ± 4*	6.38 ± 0.12*
Oua-treated	145 ± 5*$^{+}$	6.38 ± 0.05*$^{+}$	128 ± 8*	6.21 ± 0.11*
Indomethacin				
Untreated	113 ± 4	5.67 ± 0.06	51 ± 9*	5.54 ± 0.03*
Oua-treated	125 ± 6	5.63 ± 0.03	49 ± 7	5.68 ± 0.07

Values represent means ± s.e.m. *t*-test: *$P < 0.05$ vs Control; $^{+}P < 0.05$, ouabain-treated vs untreated. $N = 6–9$.

NO synthase inhibitor L-NAME (100 μM). This drug increased the potency of agonists in both arteries from both groups. The maximum response was also increased in all preparations except in mesenteric resistance arteries from untreated rats (Figure 4 and Table 1). The enhancement induced by L-NAME in α-adrenergic responses was greater in superior mesenteric than in mesenteric resistance arteries (see dAUC graph in Figure 4).

In both mesenteric resistance and superior mesenteric arteries, the potentiation induced by L-NAME was greater in arteries from ouabain-treated rats (see dAUC comparison in Figure 4).

In mesenteric resistance arteries from untreated rats, the analysis of the dAUC shows that the increase in the response to noradrenaline was larger after endothelium removal (46.7 ± 6.5) than after L-NAME incubation (25.2 ± 4.2%, $P < 0.05$); in contrast, in segments from ouabain-treated rats, the potentiation after endothelium removal (37.3 ± 3.7) or L-NAME incubation (46.6 ± 6.1%, $P > 0.05$) was similar. In superior mesenteric arteries, both endothelium removal and L-NAME similarly increased the concentration–response curve for phenylephrine in segments from untreated (dAUC: endothelium removal: 91.8 ± 10.2 vs L-NAME: 103.0 ± 16.8%, $P > 0.05$) and ouabain-treated rats (dAUC: endothelium removal: 192.0 ± 17.6 vs L-NAME: 218.0 ± 7.8%, $P > 0.05$).

Treatment with losartan did not alter the differences observed in L-NAME effects on α-adrenergic responses in both types of artery from ouabain-treated and untreated rats (Table 2).

Table 2 Effect of endothelium denudation (E−), L-NAME and indomethacin treatment on E_{max} and pD_2 to noradrenaline in MRA and to phenylephrine in SMA from rats treated with losartan (Los) or ouabain+losartan (Oua+Los) for 5 weeks

	MRA		*SMA*	
	E_{max}	pD_2	E_{max}	pD_2
Control				
Los-treated	114±1	6.07±0.02	95±2	5.78±0.09
Oua+Los-treated	114±2	6.00±0.02	76±6[+]	5.65±0.12
E−				
Los-treated	115±2	6.60±0.03*	122±4*	6.15±0.11*
Oua+Los-treated	117±3	6.56±0.10*	117±3*	6.19±0.07*
L-NAME				
Los-treated	120±2	6.34±0.05*	126±4*	6.14±0.06*
Oua+Los-treated	131±5*[+]	6.32±0.05*	120±4*	6.35±0.08*
Indomethacin				
Los-treated	110±3	5.90±0.05*	49±9*	5.26±0.12*
Oua+Los-treated	119±3	5.84±0.06*	70±8	5.73±0.13

Values represent means±s.e.m. *t*-test: *$P<0.05$ vs Control; [+]$P<0.05$, Oua+Los-treated vs Los-treated. $N=7$–8.
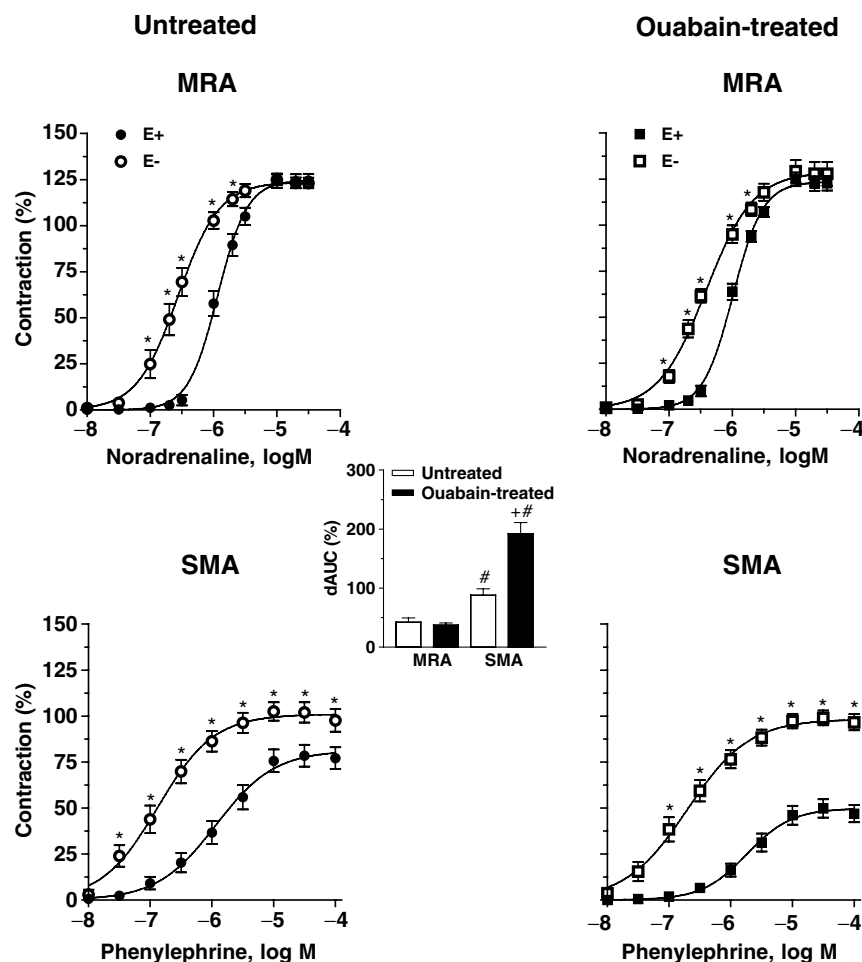

Figure 3 Effect of endothelium denudation on the concentration-dependent response curves to noradrenaline and phenylephrine in MRA and SMA, respectively, from ouabain-treated ($N=7$–9) and untreated ($N=7$–9) rats. Results (means±s.e.m.) are expressed as a percentage of response elicited by KCl. ANOVA (two-way): *$P<0.05$. The inset graph shows the dAUC to noradrenaline or phenylephrine in endothelium-intact (E+) and -denuded (E−) arteries. dAUC values (means±s.e.m.) are expressed as a percentage of the difference of the corresponding AUC for segments with intact endothelium (unpaired *t*-test, [#]$P<0.05$, SMA vs MRA; [+]$P<0.05$ ouabain-treated vs untreated rats).
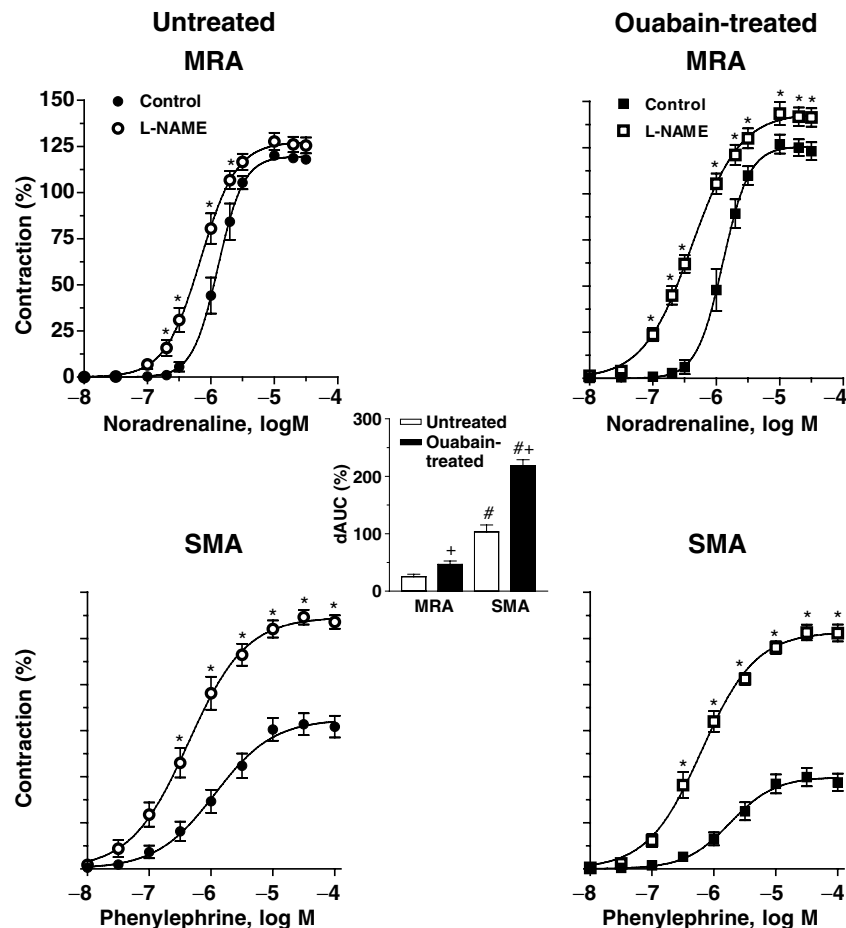
Figure 4 Effect of L-NAME ($100\,\mu$M) on the concentration-dependent response curves to noradrenaline and to phenylephrine performed in MRA and SMA from ouabain-treated ($N = 6$–9) and untreated ($N = 7$–9) rats. Results (means $\pm$ s.e.m.) are expressed as a percentage of response elicited by KCl. ANOVA (two-way): *$P < 0.05$. The inset graph shows the dAUC to noradrenaline or phenylephrine in segments in the absence and in the presence of L-NAME. dAUC values (means $\pm$ s.e.m.) are expressed as a percentage of the differences of the corresponding AUC for segments in the absence of L-NAME (unpaired *t*-test, #$P < 0.05$, SMA vs MRA; +$P < 0.05$ ouabain-treated vs untreated rats).

Effect of COX inhibition on α-adrenergic responses

As shown in Figure 5 and Table 1, indomethacin shifted the concentration–response curves for noradrenaline or phenylephrine rightward in both arteries from untreated rats; this effect was greater in superior mesenteric arteries (see dAUC graph in Figure 5). Ouabain treatment did not alter the effect of indomethacin in mesenteric resistance arteries, but abolished it in superior mesenteric arteries (Figure 5 and Table 1).

In mesenteric resistance arteries from both groups, incubation with L-NAME ($100\,\mu$M) plus indomethacin ($10\,\mu$M) shifted the concentration–response curves for noradrenaline leftward (Figure 6 and Table 3). However, this effect was larger in segments from ouabain-treated rats, as shown by the values for dAUC (Figure 6). The addition of TEA ($2\,$mM), together with L-NAME and indomethacin, produced a further leftward shift of the curve for noradrenaline in segments from untreated rats, but not in those from ouabain-treated rats (Figure 6).

Treatment with losartan modified neither the effect of indomethacin on α-adrenergic responses in mesenteric resistance and superior mesenteric arteries (Table 2) nor the effect

of indomethacin plus L-NAME plus TEA in mesenteric resistance arteries from either group (Table 3).

Western blot analysis of eNOS expression

Figure 7 shows the eNOS isoform expression detected by the Western blot technique in mesenteric resistance and superior mesenteric arteries from ouabain-treated and untreated rats. eNOS protein expression was not affected by chronic ouabain treatment in either type of artery.

Discussion

It is now established that chronic treatment with ouabain, centrally or peripherally administered, induces hypertension in rats (Huang *et al.*, 1994; Manunta *et al.*, 1994; 2001; Rossoni *et al.*, 2002a, b; Di Filippo *et al.*, 2003). This hypertension has been associated with an increased sympathetic tone that may be due to activation of the central regulatory systems involving renin–angiotensin (Huang & Leenen, 1999; Zhang & Leenen, 2001) and endothelin (Di Filippo *et al.*, 2003). Regional
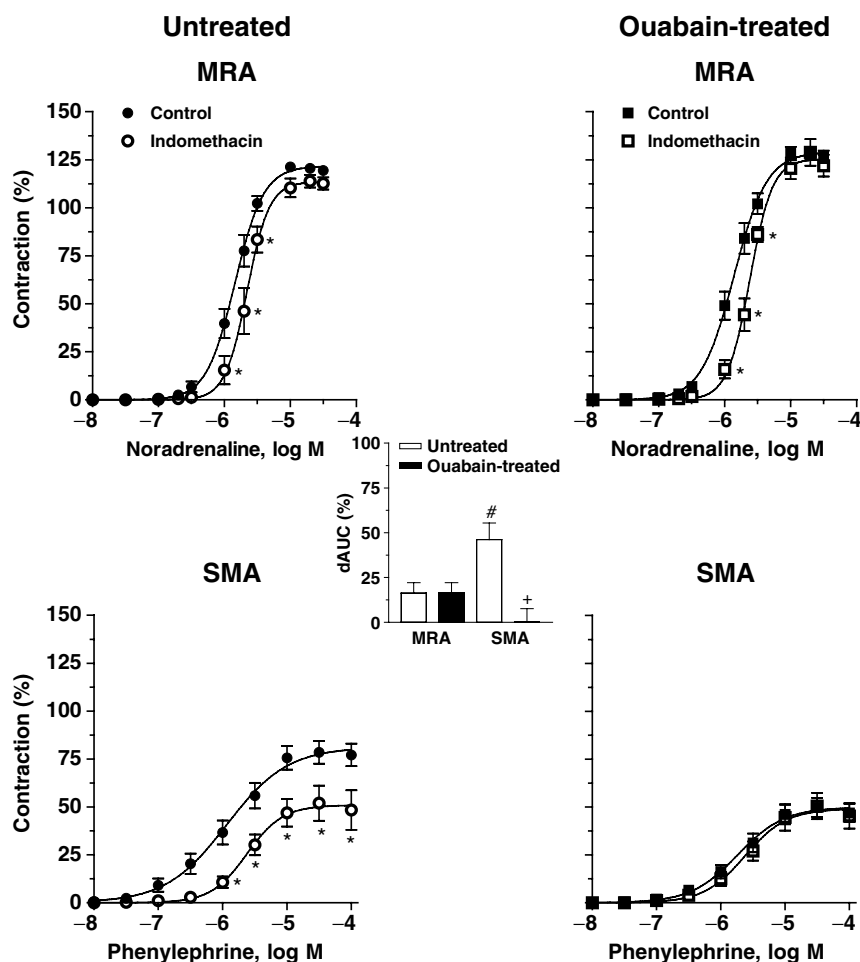
Figure 5 Effect of indomethacin (10 μM) on concentration-dependent response curves to noradrenaline and to phenylephrine performed in MRA and SMA from ouabain-treated ($N = 6$–9) and untreated ($N = 6$–9) rats. Results (means ± s.e.m.) are expressed as a percentage of response elicited by KCl. ANOVA (two-way): *$P < 0.05$. The inset graph shows the dAUC to noradrenaline or phenylephrine in segments in the absence and in the presence of indomethacin. dAUC values (means ± s.e.m.) are expressed as a percentage of the difference of the corresponding AUC for segments in the absence of indomethacin (unpaired *t*-test, #$P < 0.05$, SMA vs MRA; +$P < 0.05$ ouabain-treated vs untreated rats).
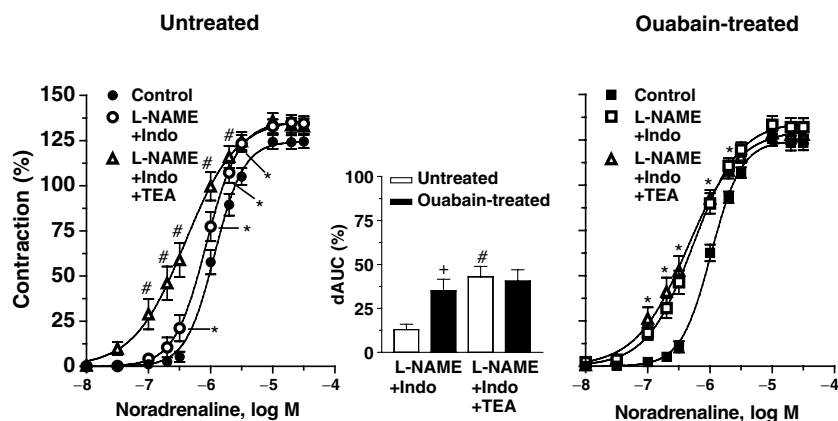

Figure 6 Effect of preincubation with L-NAME (100 μM) plus indomethacin (Indo, 10 μM) and with L-NAME plus Indo plus TEA (2 mM) on concentration–response curves to noradrenaline in mesenteric resistance arteries from untreated ($N = 8$) and ouabain-treated ($N = 8$) rats. Results (means ± s.e.m.) are expressed as a percentage of response elicited by KCl. ANOVA (two-way): *$P < 0.05$, L-NAME + Indo vs Control; #$P < 0.05$, L-NAME + Indo + TEA vs L-NAME + Indo. The inset graph shows the dAUC to noradrenaline in segments in the absence and in the presence of L-NAME + Indo and L-NAME + Indo + TEA. dAUC values (means ± s.e.m.) are expressed as a percentage of the difference of the corresponding AUC for segments in the absence of drugs (unpaired *t*-test, #$P < 0.05$, L-NAME + Indo + TEA vs L-NAME + Indo; +$P < 0.05$ ouabain-treated vs untreated rats).

Table 3 Effect of TEA treatment on pD_2 and E_{max} values to noradrenaline in endothelium intact mesenteric resistance arteries, previously incubated with L-NAME plus indomethacin (Indo), from rats treated with vehicle (untreated), ouabain, losartan or ouabain plus losartan for 5 weeks

	Control		*L-NAME + Indo*		*L-NAME + Indo + TEA*	
	E_{max}	pD_2	E_{max}	pD_2	E_{max}	pD_2
Untreated	124±3	5.93±0.04	132±4	6.11±0.06*	135±4	6.43±0.09*+
Ouabain-treated	125±4	5.98±0.03	134±5	6.30±0.04*	129±5	6.39±0.08*
Losartan-treated	109±4	6.04±0.05	114±3	6.31±0.04*	122±3	6.57±0.09*+
Ouabain + Losartan-treated	113±3	6.00±0.03	131±4	6.26±0.07*	130±4	6.29±0.09*

Values represent means±s.e.m. *t*-test: *$P<0.05$ vs Control; +$P<0.05$ vs L-NAME + Indo. $N = 7$–8.

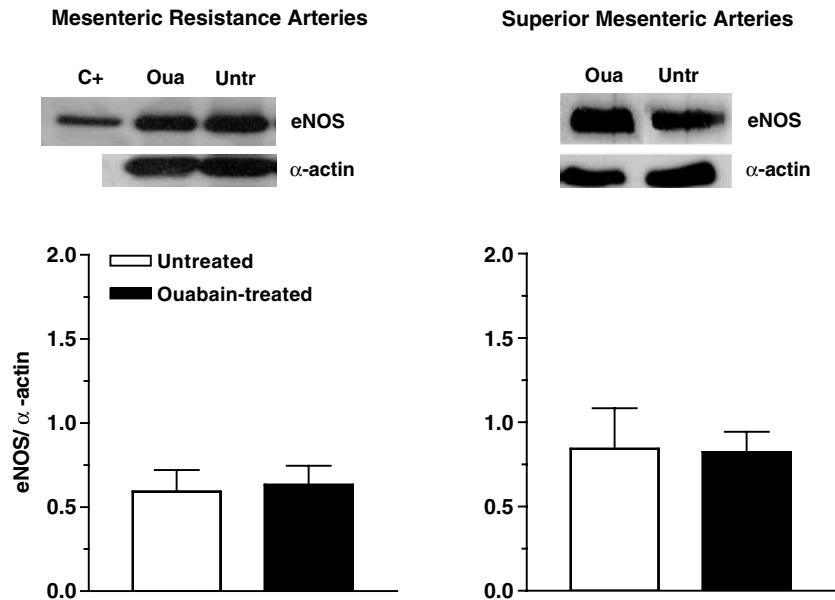**Mesenteric Resistance Arteries**   **Superior Mesenteric Arteries**

Figure 7 Upper panel: Representative blot for eNOS protein expression in mesenteric resistance and superior mesenteric arteries from ouabain-treated (Oua) and untreated rats (Untr). The first lane shows the positive control (C +) for eNOS. Lower panel: Densitometric analysis of the Western blot for eNOS protein expression. Results (means±s.e.m.) are expressed as the ratio between the signal for the eNOS protein and the signal for α-actin. The number of arterial samples used was 7–8.

differences in vascular reactivity following chronic treatment with ouabain have been reported. Reduction in the response to phenylephrine in aortae and superior mesenteric arteries (Cargnelli *et al.*, 2000; Rossoni *et al.*, 2002a, b) and no change in caudal arteries (Rossoni *et al.*, 2002a, b) have been found in this model; however, in renal arteries, contractions produced by phenylephrine and K^+ were increased (Kimura *et al.*, 2000). In addition, Di Filippo *et al.* (2003) reported increased renal vascular resistance and decreased renal blood flow, suggesting an important role of the renal vascular bed in the development and/or maintenance of the hypertension in rats chronically treated with ouabain. The reason for these regional differences is not known, but different roles of these vascular beds in the development and/or maintenance of ouabain-induced hypertension would explain them. The above-mentioned results let us to speculate that resistance and conductance vessels might respond differently to chronic ouabain treatment. The present study was designed to compare the effect of hypertension induced by chronic treatment with ouabain on the endothelial modulation of the responses mediated by α-adrenoceptor activation in the main, capacitance, and the fourth-order, resistance, branches of

the mesenteric artery and the effect of an antihypertensive treatment with an AT_1-angiotensin receptor antagonist, losartan, on these responses.

After ouabain treatment, contractile responses mediated by α-adrenoceptor activation were reduced in superior mesenteric artery, as described previously (Rossoni *et al.*, 2002a), and remained unaltered in mesenteric resistance arteries, confirming the regional differences after ouabain treatment. Both endothelium removal and NOS inhibition by L-NAME potentiated the contractile responses mediated by α-adreno-ceptors to a greater extent in the superior mesenteric artery than in the resistance mesenteric arteries. These observations suggest that the endothelial modulation of responses mediated by α-adrenoceptors is greater in mesenteric conductance than in resistance arteries.

In superior mesenteric artery, the increases in phenylephrine responses after endothelium removal or L-NAME treatment were similar and they were greater in vessels from ouabain-treated animals. This suggests that NO is the main modulator of phenylephrine responses and that chronic treatment with ouabain increases the release of endothelial NO, which would be responsible for the reduced effects of phenylephrine

observed in superior mesenteric artery, in agreement with our previous observation in rat aorta (Rossoni *et al.*, 2002b). In mesenteric resistance arteries from ouabain-treated rats, L-NAME induced a greater increment in the curves to noradrenaline than in the arteries from untreated rats, as observed in superior mesenteric artery. However, removal of the endothelium potentiated the α-adrenergic contractile responses similarly in segments from ouabain-treated and untreated rats. On the other hand, in resistance arteries from untreated rats, the effect of endothelium removal was greater than that of NO synthesis inhibition with L-NAME. Nevertheless, in segments from ouabain-treated rats, both endothelium removal and L-NAME similarly increased the curves to noradrenaline. These observations suggest that in mesenteric resistance vessels ouabain treatment increases endothelial NO modulation of α-adrenergic responses, as also occurred in superior mesenteric arteries, but decreases the participation of a non-NO mediator.

Chronic treatment with ouabain did not alter the expression of eNOS in either the mesenteric resistance or superior mesenteric arteries, discarding the involvement of upregulated eNOS expression in the increased NO production in the arteries used in this study. This is in contrast to our previous results in rat aorta from ouabain-treated animals in which we found that the increased NO modulation is accompanied by increased expression of eNOS (Rossoni *et al.*, 2002b). Nevertheless, an increase in the activity of this isoform cannot be discarded since increased eNOS activity has been associated to hypertension (Nava *et al.*, 1995; Hayakawa & Raij, 1997; Vaziri *et al.*, 1998). However, the relaxation induced by ACh remained unmodified after ouabain treatment. The mechanisms by which ouabain alters the NO release induced by α-adrenoceptor but not by muscarinic receptor activation are not known. Other investigators have demonstrated that, following its acute administration, ouabain increased basal NO release but did not affect bradykinin-stimulated NO release in rat carotid arteries (Xie *et al.*, 1993).

Previous studies reported that ouabain induces prostacyclin release from bovine endothelial cells (Nagakawa *et al.*, 1987) and enhances its production in aortic endothelial cells stimulated by ATP or bradykinin (Boeynaems & Ramboer, 1989). In addition, alterations in the synthesis of prostaglandins in hypertension have also been reported (Vanhoutte, 1996; Taddei *et al.*, 1998). In the current work, indomethacin reduced the potency but not the maximum response to noradrenaline in mesenteric resistance arteries from ouabain-treated and untreated rats. This suggests the participation of vasoconstrictor prostanoids rather than vasodilator prostanoids in the modulation of responses mediated by α-adrenoceptors in these arteries. The effect of indomethacin was similar in both groups, suggesting that ouabain-induced hypertension is not accompanied by changes in the synthesis and release of vasoconstrictor prostanoids in mesenteric resistance arteries. Opposite results have been reported in mesenteric resistance arteries from SHR, where the participation of contractile prostanoids in α-adrenergic responses has been found to be reduced (Briones *et al.*, 2002).

In superior mesenteric artery from untreated animals, indomethacin produced a nonparallel shift to the right, with a reduction in the maximum response in the concentration–response curves for phenylephrine. However, in segments from ouabain-treated rats, indomethacin did not alter phenylephr-

ine responses, suggesting that ouabain treatment causes the loss of a COX-derived positive modulator for responses mediated by α-adrenoceptors. Thus, in addition to the increase in NO release mentioned above, this mechanism might account for the reduced responses to phenylephrine in the superior mesenteric artery following chronic treatment with ouabain.

It is established that EDHF induces hyperpolarization and vasodilatation through activation of K^+ channels (Feletou & Vanhoutte, 1996). In addition, nanomolar concentrations of ouabain induce the release of an endothelium-derived relaxing factor that seems to open K_{Ca} channels in the rat-tail arterial bed preparation (Rossoni *et al.*, 1999). Therefore, the effects of TEA in mesenteric resistance arteries previously treated with L-NAME plus indomethacin were analyzed. In arteries from both groups, pretreatment with L-NAME plus indomethacin produced a leftward shift in the concentration–response curve for noradrenaline; this effect was greater in segments from ouabain-treated rats, confirming the increased modulation of NO in noradrenaline responses after ouabain treatment. Subsequent administration of TEA shifted the concentration–response curves for NE further leftward in segments from untreated rats, suggesting that EDHF is released together with NO during the smooth muscle contraction produced by NE, as reported previously (Dora *et al.*, 2000). However, TEA had no additional effect in arteries from ouabain-treated rats, suggesting that only NO mediates endothelial modulation in these arteries.

It has been described previously that the hypertensinogenic effect of chronic ouabain is associated to increased activity in sympathoexcitatory pathways and decreased activity in sympathoinhibitory pathways, and that both effects can be prevented by concomitant treatment with losartan (Zhang & Leenen, 2001). In the current work, treatment with losartan significantly reduced the arterial blood pressure in both ouabain-treated and untreated rats, abolishing differences between both groups. In spite of this, losartan treatment did not alter the following findings observed after ouabain treatment: (1) the reduced adrenergic responses in superior mesenteric artery and the unaltered response in mesenteric resistance artery; (2) the large NO participation and the impairment of the COX-dependent release of vasoconstrictor prostanoids in response to phenylephrine observed in the superior mesenteric artery; and (3) the increased modulatory effect of NO and the reduced effect of EDHF in the mesenteric resistance artery. These results allow us to suggest that the peripheral vascular alterations observed after chronic ouabain treatment are not a consequence of the elevated arterial blood pressure or the activation of the renin–angiotensin system, but are probably related to the high circulating levels of ouabain. In agreement, Cargnelli *et al.* (2000) demonstrated that chronic ouabain treatment reduced the vasoconstrictor response induced by phenylephrine in rat aorta, an effect that was independent of elevated blood pressure. On the other hand, other authors have found that chronic treatment with other cardiac glycoside such as digoxin does not raise the blood pressure (Manunta *et al.*, 2000) but normalizes the ouabain-dependent hypertension (Huang *et al.*, 1999; Manunta *et al.*, 2000). In addition, ouabain treatment, but not digoxin treatment, induced changes in renal vascular reactivity (Kimura *et al.*, 2000). These results suggest that the induction of hypertension by ouabain in rats may be independent of Na pump inhibition. Thus, new binding sites for ouabain in

adrenocortical cells have been described (Ward *et al.*, 2002), that could be involved in the vascular changes observed in this hypertension model.

The results presented here show that, in superior mesenteric artery, ouabain treatment reduces the vasoconstrictor response to phenylephrine; this may be explained by an increase in the NO pathway and an impairment of the release of contractile prostanoids. In mesenteric resistance arteries, a greater modulatory effect of endothelial NO and an impairment of EDHF participation would help to explain the unaltered noradrenaline-induced contraction after ouabain treatment. Differences in the endothelial modulation of α-adrenergic responses would explain the effect of ouabain treatment in both arteries. In this sense, differences in the participation of endothelial factors in endothelium-dependent relaxation in resistance and conductance arteries have been described (Freitas *et al.*, 2003). The different role of conductance and resistance arteries in the development and/or maintenance of ouabain-induced hypertension would be also implicated. In any case, these results highlight that extrapolations from conductance to resistance vessels may not be appropriate when hypertension is studied. Although losartan prevented the development of ouabain-induced hypertension, the above-noted vascular alterations were still observed. In conclusion, chronic ouabain treatment produces different effects on the vascular reactivity induced by α-adrenoceptor activation in rat conductance and resistance mesenteric arteries, and these effects are independent of the elevated arterial blood pressure or the renin–angiotensin system.

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