

# Contribution of the endothelin and renin–angiotensin systems to the vascular changes in rats chronically treated with ouabain

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**1** Renin–angiotensin and endothelin systems are involved in the cardiovascular effects produced by treatment with ouabain. We recently demonstrated that the contractile response to phenylephrine is decreased in ouabain-treated rats. The present study investigated whether endothelin-1 (ET-1) and angiotensin II (Ang II) contributes to the vascular changes observed in rats chronically treated with ouabain.

**2** Wistar rats were treated with ouabain ( $8.0 \mu\text{g day}^{-1}$ , s.c. pellets for 5 weeks) alone or in combination with an endothelin type A receptor (ET<sub>A</sub>) antagonist, BMS182874 ( $40 \text{ mg kg}^{-1} \text{ day}^{-1}$ , per gavage) or an angiotensin type 1 (AT<sub>1</sub>) receptor antagonist, losartan ( $15 \text{ mg kg}^{-1} \text{ day}^{-1}$ , p.o.).

**3** Treatment with ouabain increased systolic blood pressure and treatment with either losartan or BMS182874 prevented the development of ouabain-induced hypertension.

**4** The sensitivity and maximal response for phenylephrine were reduced in aortic rings from ouabain-treated rats. Removal of the endothelium or *in vitro* exposure to an inhibitor of nitric oxide synthase (NOS), *N*-nitro-L-arginine methyl ester (L-NAME,  $100 \mu\text{M}$ ) increased the responses to phenylephrine, an effect that was more pronounced in aortas from ouabain-treated rats. Endothelial NOS protein (eNOS) expression was increased after ouabain treatment. Treatment with BMS182874, but not with losartan, prevented the effects of ouabain on the reactivity of phenylephrine and in eNOS protein expression.

**5** Gene expression of pre-pro-ET-1 and ET<sub>A</sub> receptors was increased in aortic rings from ouabain-treated rats. ET<sub>B</sub> receptor gene expression was not altered by ouabain treatment.

**6** In conclusion, our results suggest that endothelin and angiotensin systems play an important role in the development of ouabain-induced hypertension. However, ET-1, by activation of ET<sub>A</sub> receptors, but not Ang II, contributes to changes in vascular reactivity to phenylephrine induced by chronic treatment with ouabain.

*British Journal of Pharmacology* (2004) **143**, 794–802. doi:10.1038/sj.bjp.0705994

**Keywords:** Ouabain; hypertension; endothelium; nitric oxide; renin–angiotensin system; endothelin

**Abbreviations:** Ang II, angiotensin II; AT<sub>1</sub>, angiotensin type 1 receptor; dAUC, difference of area under the concentration–response curves;  $E_{\text{max}}$ , maximum response; eNOS, endothelial nitric oxide synthase; ET-1, endothelin-1; ET<sub>A</sub>, endothelin type A receptor; ET<sub>B</sub>, endothelin type B receptor; KHB, Krebs–Henseleit bicarbonate buffer; L-NAME, *N*-nitro-L-arginine methyl ester; NO, nitric oxide; PAGE, polyacrylamide gel;  $pD_2$ , negative logarithm of concentrations producing 50% of maximum response; RT–PCR, reverse transcriptase–polymerase chain reaction; SBP, systolic blood pressure; SDS, sodium lauryl sulphate

## Introduction

There is extensive experimental evidence supporting an important role for the endogenous cardiac glycoside ouabain on the pathogenesis of hypertension (Hamlyn & Manunta, 1992; Hamlyn *et al.*, 1996). One of the most important observations that support this affirmation is that plasma ouabain levels are increased in some forms of hypertension, as well as in human essential hypertension (Overbeck *et al.*, 1976;

Hamlyn *et al.*, 1982; 1996). In addition, the acute or chronic administration of ouabain or its related isomers to normotensive rats increases blood pressure (Huang *et al.*, 1994; Manunta *et al.*, 1994; 2001; Kimura *et al.*, 2000; Rossoni *et al.*, 2001; 2002a, b; D’Amico *et al.*, 2003; Di Filippo *et al.*, 2003; Xavier *et al.*, 2004a, b). However, conflicting results showing that hypertension is accompanied by normal plasma ouabain levels (Doris, 1994; Gonick *et al.*, 1998; Priyadarshi *et al.*, 2003; Wang *et al.*, 2003) or that ouabain is ineffective to produce hypertension (Li *et al.*, 1995; Pidgeon *et al.*, 1996; Cargnelli *et al.*, 2000) have also been published.

Both central and peripheral mechanisms are involved in the production of hypertension by ouabain. Chronic treatment

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Advance online publication: 11 October 2004

with ouabain increases the content of ouabain in some brain areas related to cardiovascular regulation (Huang *et al.*, 1994). The interplay of the cardiac glycoside with some excitatory components present in these areas is one of the mechanisms that explain the genesis of hypertension in this model (Huang *et al.*, 1994; Huang & Leenen, 1999; Zhang & Leenen, 2001; Di Filippo *et al.*, 2003). The central renin–angiotensin and endothelin systems are examples of these sympathoexcitatory components involved in the hypertensinogenic effect of ouabain, since both the sympathetic hyperactivity and hypertension induced by chronic treatment with ouabain are prevented by angiotensin type 1 (AT<sub>1</sub>) and endothelin-1 (ET-1) type A (ET<sub>A</sub>) receptors blockade (Zhang & Leenen, 2001; Di Filippo *et al.*, 2003).

In addition to central mechanisms, peripheral mechanisms may contribute to the elevated arterial blood pressure induced by chronic ouabain treatment (Kimura *et al.*, 2000; Di Filippo *et al.*, 2003). However, it is possible that changes in peripheral mechanisms may play a compensatory role or may counteract ouabain-induced hypertension (Rossoni *et al.*, 2002a, b; Xavier *et al.*, 2004a, b). Cargnelli *et al.* (2000) demonstrated that phenylephrine-induced contraction was reduced while ET-1-induced contraction was increased in rat aortae after long-term treatment with ouabain. We have also demonstrated that in this hypertension model the vascular reactivity to phenylephrine is decreased and that this is accompanied by an increased activity and expression of the sodium–potassium ATPase pump (Na<sup>+</sup>, K<sup>+</sup>-ATPase) (Rossoni *et al.*, 2002a). These alterations are also associated with increased endothelium-derived nitric oxide (NO) production and increased expression of the endothelial nitric oxide synthase (eNOS) and neuronal isoforms of NOS (Rossoni *et al.*, 2002b).

Based on these observations, the purpose of the present work was to investigate if the endothelin and/or renin–angiotensin systems contribute to the vascular changes in rats chronically treated with ouabain. Therefore, we evaluated if treatment with losartan, an AT<sub>1</sub>–angiotensin-receptor antagonist, or BMS182874, a selective ET<sub>A</sub>-receptor antagonist, would prevent the changes in aortic reactivity produced by chronic treatment with ouabain. Expression of the genes for pre-pro-ET-1, ET<sub>A</sub> and ET<sub>B</sub> receptors in aorta were also evaluated.

## Methods

Male Wistar rats (6 weeks old) were obtained from colonies maintained at the Animal Quarters of the Institute of Biomedical Sciences of the University of Sao Paulo and Facultad de Medicina of the Universidad Autónoma de Madrid. Rats were housed at a constant room temperature, humidity and 12 h light/dark cycle and had free access to tap water and standard rat chow. All experiments comply with the guidelines for biomedical research as stated by the Brazilian Societies of Experimental Biology and with the current Spanish and European laws (RD 223/88 MAPA and 609/86).

Rats were anaesthetized 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, U.S.A.) containing ouabain (0.5 mg pellet<sup>-1</sup>) or vehicle (placebo). Animals were divided into six groups: vehicle, ouabain (approximately 8.0 µg day<sup>-1</sup>),

Vehicle plus losartan (15 mg kg<sup>-1</sup> day<sup>-1</sup>, p.o.), Ouabain plus losartan, Vehicle plus BMS182874 (40 mg kg<sup>-1</sup> day<sup>-1</sup>, p.o. per gavage) and Ouabain plus BMS182874. 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. The dose of losartan was chosen in order to produce a reduction of approximately 40 mmHg in the systolic blood pressure (SBP) of ouabain-treated rats (Bravo *et al.*, 2001). The dose of the ET<sub>A</sub> antagonist BMS 182874 was chosen based on previous works from our group (Callera *et al.*, 2003). SBP was measured weekly in awake animals by the tail-cuff method (Buñag, 1973) before the beginning of treatment and once a week during treatment until week 5.

### Vascular reactivity study

After 5 weeks, animals were anaesthetized with diethyl ether and killed by exsanguination. The thoracic aorta was removed and placed in cold oxygenated Krebs–Henseleit bicarbonate buffer (KHB). The buffer consisting of (in mM): NaCl 118; KCl 4.7; NaHCO<sub>3</sub> 25; CaCl<sub>2</sub>·2H<sub>2</sub>O 2.5; KH<sub>2</sub>PO<sub>4</sub> 1.2; MgSO<sub>4</sub>·7H<sub>2</sub>O 1.2; glucose 11 and ethylenediamine-tetraacetic acid 0.01. Segments of thoracic aorta (3 mm in length), free of fat and connective tissue, were mounted between two steel hooks in isolated tissue chambers containing gassed (95% O<sub>2</sub> and 5% CO<sub>2</sub>) KHB, at 37°C, under a resting tension of 1 g. Isometric tension was recorded by using an isometric force displacement transducer connected to an acquisition system (PowerLab 8/S, ADInstruments Pty Ltd, Castle Hill, Australia).

After a 45 min equilibration period, each aortic ring was exposed twice to KCl (75 mM), to assess its maximum contractility. After 30 min, rings were contracted with a concentration of phenylephrine that induced approximately 50% of the KCl contraction, and then acetylcholine (10 µM) was added to assess the integrity of the endothelium. A relaxation equal to or greater than 90% was considered as evidence of the functional integrity of the endothelium.

After 60 min, cumulative concentration–response curves for phenylephrine (0.1 nM–30 µM) were generated. In some experiments, the 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 relaxation induced by acetylcholine (10 µM) in rings precontracted with phenylephrine. Additionally, the effect of the nonselective NOS inhibitor *N*-nitro-L-arginine methyl ester (L-NAME, 100 µM) on concentration–response curves for phenylephrine was investigated. L-NAME was added 30 min before generating the concentration–response curve.

### Western blot analysis of eNOS protein expression

Proteins from homogenized aortae (30 µg per lane) were separated by 7.5% sodium lauryl sulphate–polyacrylamide gel (SDS–PAGE) and transferred to polyvinylidene difluoride membranes overnight. Next, the membranes were incubated for 1 h at room temperature with mouse monoclonal antibody for eNOS (1:2500, Transduction Laboratories, Lexington, U.K.), and after washing were incubated with antimouse IgG antibody conjugated to horseradish peroxidase (1:2000, Transduction Laboratories, U.K.). 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, U.S.A.). Signals on the immunoblot were quantified using a computer program (NIH Image V1.56, U.S.A.). The same membranes were used to determine  $\alpha$ -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.

#### Reverse transcriptase–polymerase chain reaction (RT–PCR)

Total cell RNA was isolated from aortae using Trizol Reagent (Gibco BRL, Life Technologies, Rockville, MD, U.S.A.). Total RNA (20 ng per sample) was used for RT in the presence of an RNAase inhibitor 20 U (RNAase OUT, Invitrogen Life Technologies), 200 U of Superscript II RT (Invitrogen, Carlsbad, CA, U.S.A.) and 0.5  $\mu$ g of oligo-(dT) 12–18 primer at 42°C for 50 min, according to the manufacturer's specifications. The cDNA was stored at –20°C until required for the PCR. PCR primers were designed as follows: pre–pro-ET-1, antisense primer CTCGCTCTATGTAAGTCATGG, sense primer GCTCCTGCTCCTCTTGATG (471-bp fragment); ET<sub>A</sub> receptor, antisense primer CTGTGCTGCTCGCCCTTGTA, sense primer GAAGTCGTCCGTGGGCATCA (216-bp fragment); ET<sub>B</sub> receptor, antisense primer CACGATGAGGACAATGAGAT, sense primer TTACAAGACAGCAAAGACT (565-bp fragment); and GAPDH, antisense primer CACCACCCTGTTGCTGTA, sense primer TATGATGACATCAAGAAGGTGG (219-bp fragment). GAPDH was used as an internal control for the coamplification. The following conditions were selected for PCR in a volume of 25  $\mu$ l: RT products from 20 ng of RNA, 1 U of Platinum<sup>®</sup> Taq DNA polymerase (Invitrogen), 28 cycles of amplification for pre–pro-ET-1, 32 cycles for ET<sub>A</sub> and 30 for ET<sub>B</sub> receptor genes, and 24 cycles for the GAPDH gene. Amplification was carried out using an initial denaturing cycle at 94°C for 5 min and the subsequent cycles as follows: denaturation, 30 s at 94°C; annealing, 30 s at 64°C for pre–pro-ET-1, 58°C for ET<sub>B</sub>, 66°C for ET<sub>A</sub> or 62°C for GAPDH; and extension, 45 s at 72°C. PCR products (10  $\mu$ l per lane) were electrophoresed using 1% agarose gel containing ethidium bromide (0.5  $\mu$ g ml<sup>-1</sup>). The gel was subjected to ultraviolet light and photographed. The band intensities were measured using a software package (Kodak Digital Science, Eastman Kodak Company, New Haven, CT, U.S.A.).

#### Solutions, drugs and statistical analysis

Phenylephrine hydrochloride, acetylcholine chloride and L-NAME dihydrochloride were purchased from Sigma (St Louis, MO, U.S.A.). Stock solutions (10 mM) of drugs were made in distilled water and were kept at –20°C, and appropriate dilutions were made on the day of the experiment. Losartan and BMS182874 were a gift of Merck Sharp & Dohme and of Bristol Myers Squibb Co., respectively.

All values are expressed as means  $\pm$  s.e.m. Contractile responses are expressed as a percentage of the maximum response produced by 75 mM KCl. For each concentration–response curve, the maximum effect ( $E_{max}$ ) and the concentra-

tion of agonist that produced half of  $E_{max}$  (log EC<sub>50</sub>) were estimated using nonlinear regression analysis (GraphPad Prism Software, San Diego, CA, U.S.A.). The sensitivity of the agonist is expressed as negative logarithm of concentrations producing 50% of maximum response (pD<sub>2</sub>) (–log EC<sub>50</sub>). In order to compare the magnitude of the effects of endothelium denudation and NOS blockade on the responses to phenylephrine, some results were expressed as 'differences' of area under the concentration–response curves (dAUC) in control and experimental situations. AUCs were calculated from the individual concentration–response curve plot 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  $\alpha$ -actin. Results of pre–pro-ET-1, ET<sub>A</sub> and ET<sub>B</sub> mRNA expressions are expressed relative to the intensity of GAPDH.

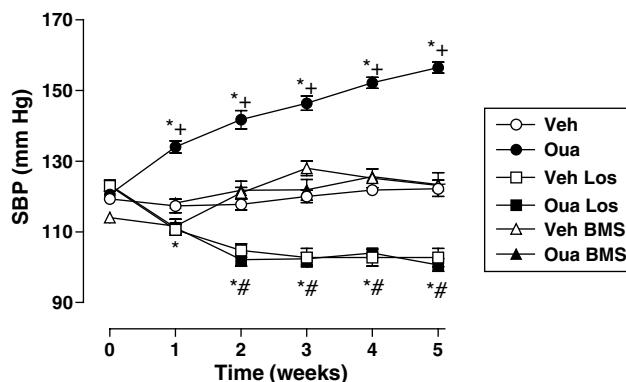
Results were analysed using Student's *t*-test and by completely randomized two-way ANOVA for comparison between groups. When ANOVA showed a significant treatment effect, Bonferroni's *post hoc* test was used to compare individual means. Differences were considered statistically significant at  $P < 0.05$ .

## Results

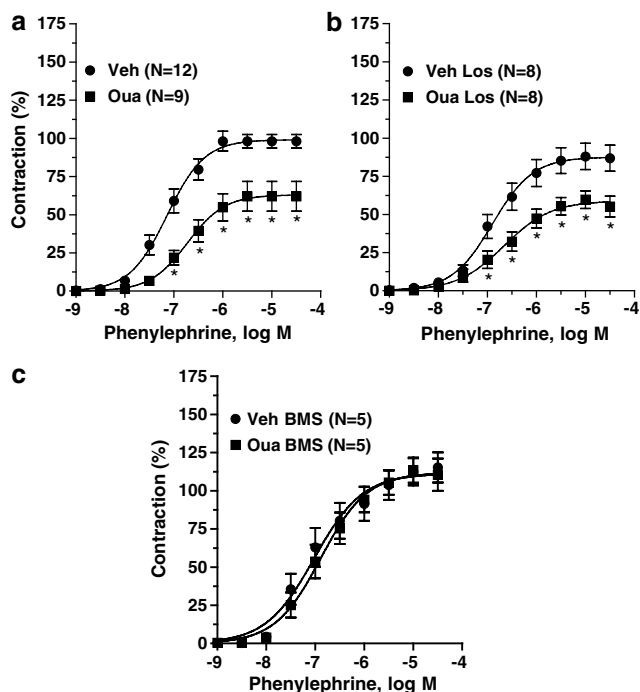
Figure 1 shows the SBP for all groups over the course of treatment. An increase, from the first week of treatment, was observed in ouabain-treated rats compared to vehicle group. In ouabain and vehicle groups, treatment with losartan significantly reduced SBP to similar levels. Treatment with BMS182874 did not change SBP in the vehicle group but prevented the development of hypertension in ouabain-treated animals. Body weight gain during the 5 weeks of treatment was similar in all groups (data not shown).

#### Vascular reactivity study

To assess the maximum contraction for each preparation, rings were exposed to 75 mM KCl. Vasoconstrictor responses to this depolarizing solution were similar in aortae from ouabain-



**Figure 1** SBP in Wistar rats treated with vehicle (Veh,  $N = 13$ ), ouabain (Oua,  $N = 21$ ), Vehicle plus losartan (Veh Los,  $N = 7$ ), Ouabain plus Losartan (Oua Los,  $N = 8$ ), Vehicle plus BMS182874 (Veh BMS,  $N = 7$ ) or Ouabain plus BMS182874 (Oua BMS,  $N = 8$ ). Results are expressed as mean  $\pm$  s.e.m. \* $P < 0.05$  vs 0 week; # vs 0 week Los group; +  $P < 0.05$  vs vehicle.



**Figure 2** Contractile responses to phenylephrine in thoracic aortic rings from vehicle (Veh) and ouabain-treated (Oua) rats (a); vehicle losartan-treated (Veh Los) and Ouabain plus losartan-treated rats (Oua Los) (b); vehicle BMS184874-treated rats (Veh BMS) and Ouabain plus BMS184874-treated rats (Oua BMS) (c). Results (means  $\pm$  s.e.m.) are expressed as a percentage of response elicited by KCl. ANOVA (two-way): \* $P < 0.05$  vs vehicle. Number of animals used is indicated within parentheses.

treated rats compared to vehicle group (ouabain:  $24.9 \pm 1.6$  vs vehicle:  $24.7 \pm 1.5$  mN,  $N = 9$  and  $12$ , respectively,  $t$ -test,  $P > 0.05$ ). This response was not altered by treatment with losartan (ouabain losartan:  $25.4 \pm 1.5$  vs vehicle losartan:  $24.7 \pm 1.2$  mN,  $N = 8$ ,  $t$ -test,  $P > 0.05$ ) or BMS182874 (ouabain BMS:  $21.0 \pm 0.9$  vs vehicle BMS:  $21.2 \pm 1.4$  mN,  $N = 5$ ,  $t$ -test,  $P > 0.05$ ).

As previously demonstrated, both the  $E_{\max}$  and  $pD_2$  for phenylephrine were reduced in intact aortic rings obtained from animals treated for 5 weeks with ouabain (Figure 2a and Table 1). Treatment with losartan did not change the concentration–response curve for phenylephrine and did not alter the effects of ouabain treatment on the phenylephrine response (Figure 2b and Table 1). In contrast to losartan, treatment with BMS182874 prevented the effects of ouabain on the actions of phenylephrine (Figure 2c and Table 1).

#### Effect of endothelium removal or NO synthesis blockade on phenylephrine responses

Endothelial denudation or treatment with L-NAME increased the maximal response and sensitivity to phenylephrine in aorta obtained from both ouabain and vehicle groups (Figures 3a, b and 4a, b). In both cases, the increase was larger in ouabain-treated rats, as shown by the values for dAUC (see inset graph in Figures 3a, b and 4a, b). The concentration–response curves for phenylephrine in aorta from the ouabain group and the vehicle group in the presence of L-NAME or following endothelium removal were not different (Table 1).

**Table 1** Changes in  $E_{\max}$  (expressed as a percentage of the response to 75 mM KCl) and  $pD_2$  to phenylephrine in thoracic aorta with (E+) and without (E-) endothelium and after L-NAME treatment from Wistar rats treated with vehicle ( $N = 5-9$ ), ouabain ( $N = 5-12$ ), Vehicle plus losartan ( $N = 8-9$ ), Ouabain plus Losartan ( $N = 8-12$ ), vehicle plus BMS182874 ( $N = 4-5$ ) or Ouabain plus BMS182874 ( $N = 4-5$ )

|                     | $E_{\max}$ (%)   | $pD_2$           |
|---------------------|------------------|------------------|
| Vehicle             |                  |                  |
| E+                  | $93.0 \pm 4.7$   | $7.17 \pm 0.1$   |
| E-                  | $119 \pm 7.7^+$  | $7.79 \pm 0.1^+$ |
| L-NAME              | $136 \pm 1.3^+$  | $7.48 \pm 0.1^+$ |
| Ouabain             |                  |                  |
| E+                  | $62.3 \pm 9.8^*$ | $6.65 \pm 0.1^*$ |
| E-                  | $126 \pm 7.8^+$  | $7.78 \pm 0.1^+$ |
| L-NAME              | $140 \pm 7.0^+$  | $7.46 \pm 0.1^+$ |
| Vehicle + losartan  |                  |                  |
| E+                  | $87.5 \pm 8.1$   | $6.90 \pm 0.1$   |
| E-                  | $108 \pm 4.5^+$  | $7.45 \pm 0.1^+$ |
| L-NAME              | $116 \pm 3.4^+$  | $7.34 \pm 0.1^+$ |
| Ouabain + losartan  |                  |                  |
| E+                  | $58.6 \pm 6.2^*$ | $6.50 \pm 0.1^*$ |
| E-                  | $107 \pm 5.0^+$  | $7.36 \pm 0.1^+$ |
| L-NAME              | $115 \pm 2.7^+$  | $7.17 \pm 0.1^+$ |
| Vehicle + BMS182874 |                  |                  |
| E+                  | $111 \pm 7.8$    | $6.82 \pm 0.1$   |
| E-                  | $142 \pm 1.5^+$  | $7.23 \pm 0.1^+$ |
| L-NAME              | $143 \pm 10.3^+$ | $7.43 \pm 0.1^+$ |
| Ouabain + BMS182874 |                  |                  |
| E+                  | $110 \pm 7.8$    | $6.82 \pm 0.1$   |
| E-                  | $148 \pm 4.6^+$  | $7.37 \pm 0.1^+$ |
| L-NAME              | $154 \pm 15.4^+$ | $7.50 \pm 0.1^+$ |

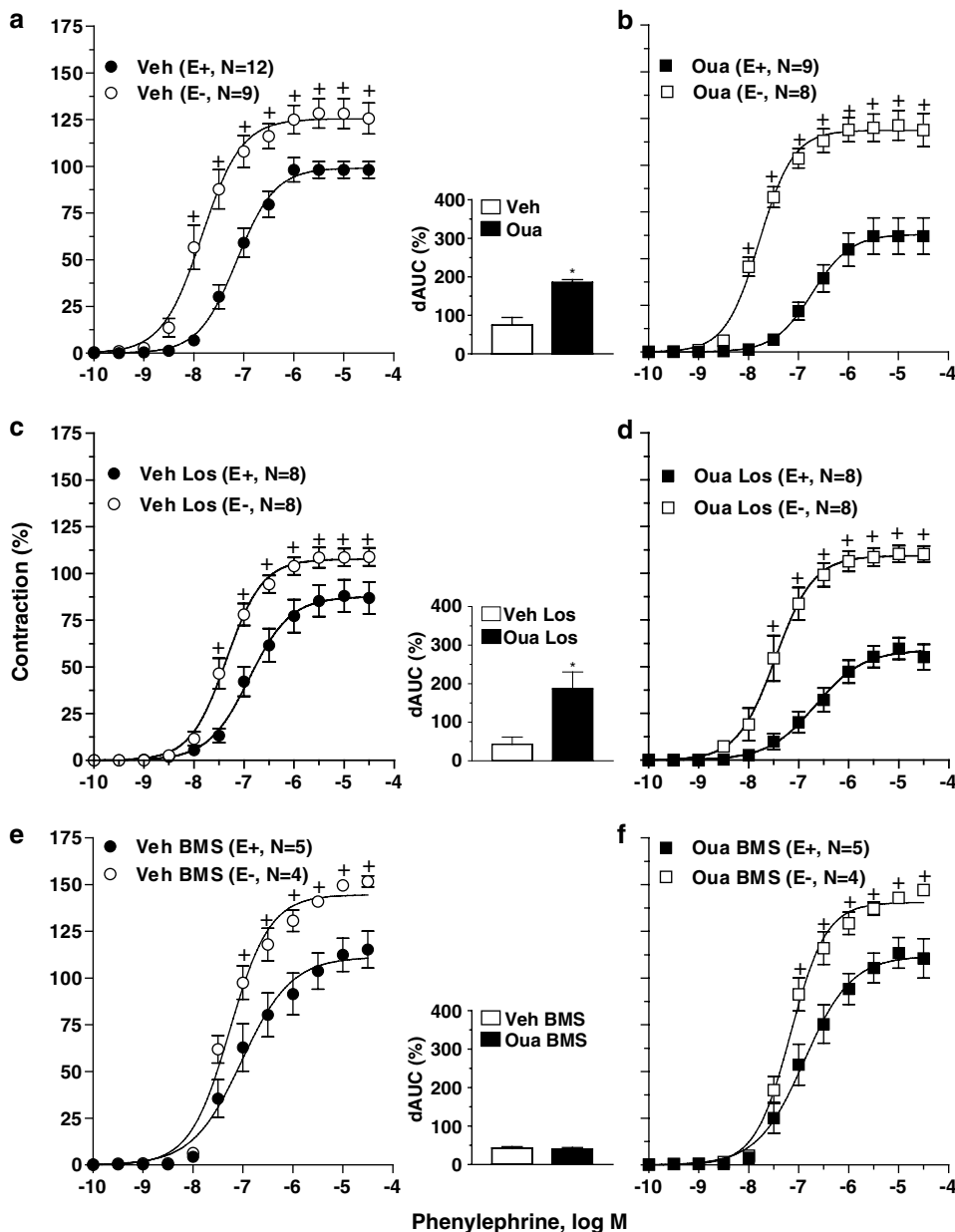
$t$ -Test, \* $P < 0.05$  vs vehicle and  $^+P < 0.05$  vs E+.

Similar changes were observed in aortae from the losartan groups (Figures 3c, d and 4c, d). Namely, the shifts in concentration–response curves for phenylephrine were greater in aorta from ouabain-treated animals in the presence of L-NAME or following endothelial denudation (see inset graph in Figures 3c, d and 4c, d). In aortae from rats treated with losartan, there were no differences in the concentration–response curves for phenylephrine in the presence of L-NAME or after endothelium damage (Table 1).

However, treatment with BMS182874 prevented the effects of ouabain on the actions of phenylephrine and abolished the differential effect of ouabain on the modulation of the endothelium and NO on the actions of phenylephrine (see dAUC graph in Figures 3e, f and 4e, f). In endothelium-denuded or L-NAME-treated aortae from rats that received BMS182874, the responses to this  $\alpha_1$ -adrenergic agonist were similar in ouabain and vehicle groups (Table 1).

#### Pre-pro-ET-1, $ET_A$ and $ET_B$ mRNA expression

Figure 5 shows results of mRNA expression, obtained by RT-PCR, for pre-pro-ET-1,  $ET_A$  and  $ET_B$  receptors in aortae from ouabain and vehicle groups. Expression of pre-pro-ET-1



**Figure 3** Effect of endothelium denudation on the concentration-dependent response curves to phenylephrine in thoracic aortic rings from vehicle (Veh) and ouabain-treated (Oua) rats (a and b); vehicle losartan-treated (Veh Los) and Ouabain plus losartan-treated rats (Oua Los) (c and d); vehicle BMS184874-treated rats (Veh BMS) and Ouabain plus BMS184874-treated (Oua BMS) rats (e and f). Results (means  $\pm$  s.e.m.) are expressed as a percentage of response elicited by KCl. ANOVA (two-way): +  $P < 0.05$  vs E+. Inset graph shows dAUC to phenylephrine in endothelium-intact (E+) and -denuded (E-) arteries. dAUC values (means  $\pm$  s.e.m.) are expressed as a percentage of the difference of the corresponding AUC for segments with intact endothelium (unpaired *t*-test, \* $P < 0.05$  vs vehicle). Number of animals used is indicated within parentheses.

mRNA was significantly increased in aortae from ouabain-treated rats. Similarly, increased  $ET_A$  receptor mRNA expression was observed in aortae from ouabain-treated rats compared to vehicle rats.  $ET_B$  receptor mRNA expression remained unchanged after chronic treatment with ouabain.

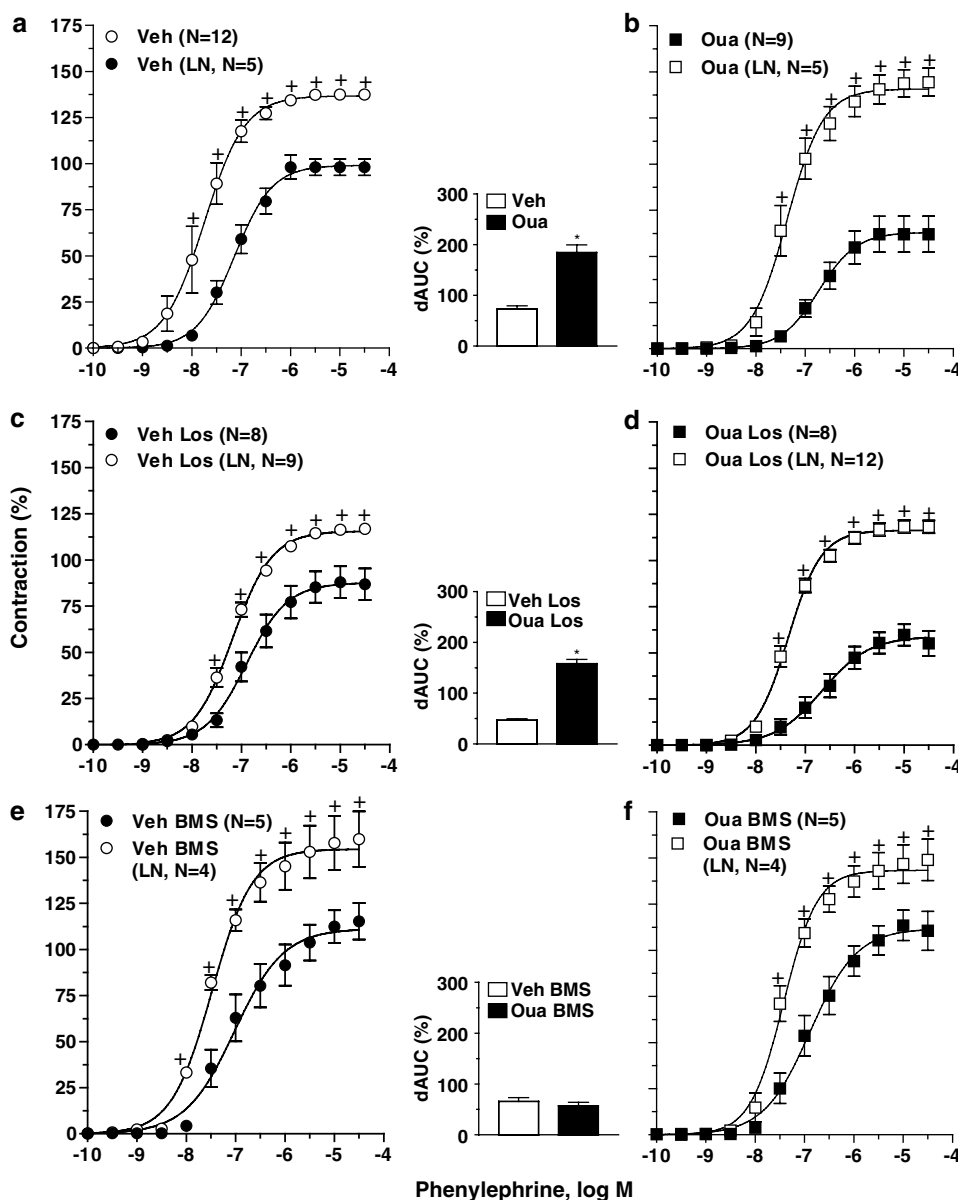
#### eNOS protein expression

eNOS expression was evaluated in homogenates of aortae obtained from all experimental groups (Figure 6). In arteries from ouabain and Ouabain plus losartan groups, eNOS expression was increased compared to that in their respective

control groups (Figure 6a and b). However, no differences were observed in eNOS expression in the aortae from vehicle and ouabain rats treated with BMS182874 (Figure 6c).

## Discussion

The present study shows that although ET-1 and angiotensin II (Ang II) contribute to the hypertension induced by chronic ouabain treatment (Huang & Leenen, 1999; Zhang & Leenen, 2001; Di Filippo *et al.*, 2003), only ET-1 contributes to changes in vascular reactivity in aortae from ouabain hypertensive rats.



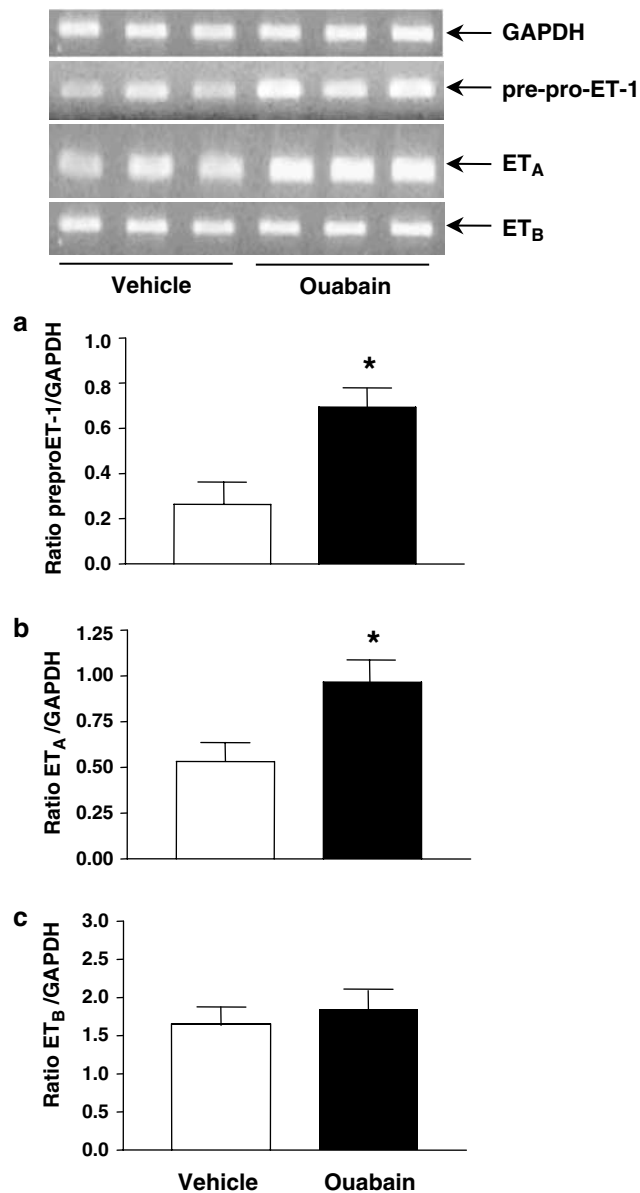
**Figure 4** Effect of L-NAME (LN, 100  $\mu$ M) on the concentration-dependent response curves to phenylephrine performed in thoracic aortic rings from vehicle (Veh) and ouabain-treated (Oua) rats (a and b); vehicle losartan-treated (Veh Los) and Ouabain plus losartan-treated rats (Oua Los) (c and d); vehicle BMS184874-treated rats (Veh BMS) and Ouabain plus BMS184874-treated rats (Oua BMS) (e and f). Results (means  $\pm$  s.e.m.) are expressed as a percentage of response elicited by KCl. ANOVA (two-way): +  $P < 0.05$  vs without L-NAME. Inset graph shows dAUC to phenylephrine in segments in the absence and 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, \* $P < 0.05$  vs vehicle). Number of animals used is indicated within parentheses.

Our data reinforce earlier observations on the effects of ouabain increasing blood pressure in rats, reducing the contractile actions of phenylephrine and producing an increase in the negative modulation of vascular reactivity by the endothelium (Huang *et al.*, 1994; Manunta *et al.*, 1994; 2001; Kimura *et al.*, 2000; Rossoni *et al.*, 2002a, b; Di Filippo *et al.*, 2003; Xavier *et al.*, 2004a, b).

It has been reported that acutely central administration of ouabain causes sympathoexcitatory and pressor effects in rats (Huang & Leenen, 1996; D'Amico *et al.*, 2003). In addition, following its chronic administration, ouabain accumulates in brain areas related to cardiovascular regulation (Huang *et al.*, 1994; Manunta *et al.*, 1994) where it might induce activation of

sympathoexcitatory outflow (Huang *et al.*, 1994; Huang & Leenen, 1999; Zhang & Leenen, 2001) and by this way increases peripheral vascular resistance and induces hypertension (Di Filippo *et al.*, 2003). On the other hand, this hypertension induced by ouabain is also accompanied by vascular compensatory mechanisms (Rossoni *et al.*, 2002a, b; Xavier *et al.*, 2004a, b), which seems to be independent of elevated arterial blood pressure (Xavier *et al.*, 2004a). This compensatory mechanism could be a consequence of a direct vascular action of ouabain and/or an adaptive response to the increased sympathetic outflow.

The central renin-angiotensin system is one of the sympathoexcitatory components involved in the hypertensinogenic



**Figure 5** Representative RT-PCR products of 3  $\mu$ g total RNA extracted from aortae of vehicle and ouabain-treated rats. Bar graphs show the densitometric values for gene expression of pre-pro-ET-1 (a), ET<sub>A</sub> (b) and ET<sub>B</sub> (c) subtype receptors. Values were normalized by the corresponding RT-PCR products for GAPDH ( $N = 4-6$ ). \* $P < 0.05$  vs vehicle.

actions of ouabain. This is shown by the demonstration that treatment with an AT<sub>1</sub>-receptor antagonist prevents the increase in central sympathetic outflow and hypertension produced by the long-term administration of ouabain (Huang & Leenen, 1999; Zhang & Leenen, 2001). In addition, the acute hypertensive effect of ouabain is attenuated in transgenic rats that are deficient in brain angiotensinogen (Huang *et al.*, 2001). In agreement with these observations, the results presented here show that AT<sub>1</sub> receptor blockade with losartan prevented the development of hypertension in ouabain-treated rats.

Although treatment with losartan prevented the development of hypertension produced by ouabain, it did not prevent the ability of ouabain to alter either the vascular actions of phenylephrine, the increased negative endothelial modulation

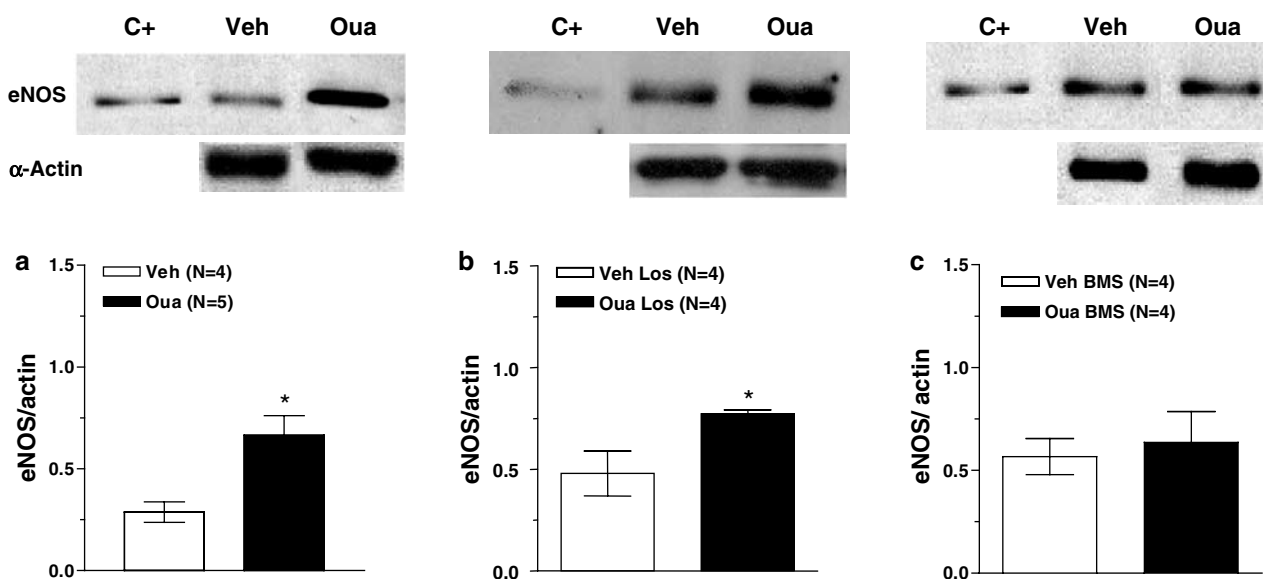
of vascular reactivity or the increased eNOS expression. Similar results were recently obtained with the effects of treatment with losartan on the vascular reactivity of conductance and resistance mesenteric arteries obtained from ouabain hypertensive rats (Xavier *et al.*, 2004a). These results suggest that Ang II *via* AT<sub>1</sub> receptors activation is involved in the genesis of hypertension but not in vascular reactivity changes caused by chronic treatment with ouabain. These two actions appear to be mediated by different pathways.

In addition, treatment with BMS182874, an ET<sub>A</sub>-receptor antagonist, also prevented the development of hypertension in chronically ouabain-treated rats. This result is in agreement with that of Di Filippo *et al.* (2003), showing that treatment with a selective ET<sub>A</sub>-receptor antagonist prevented the increase on renal vascular resistance and blood pressure in chronically ouabain-treated rats. Similarly, results from D'Amico *et al.* (2003) demonstrated that acute ouabain administration on intraperiaqueductal grey area increases total peripheral resistance and blood pressure, probably through an activation of brain ET-1. Altogether, these results suggest an important role of endothelin system on the hypertensinogenic effect of ouabain.

The endothelin system also contributes to the genesis of hypertension and to the cardiovascular changes in volume expansion-dependent models of hypertension, such as deoxycorticosterone acetate (DOCA)-salt hypertension (Tostes *et al.*, 2000; Callera *et al.*, 2003; Iglarz & Schiffrin, 2003). Salt retention and plasma volume expansion may stimulate the release of endogenous ouabain that, in turn, would stimulate the release of ET-1 from endothelial cells (Overbeck *et al.*, 1976; Yamada *et al.*, 1990; Hamlyn & Manunta, 1992; Hamlyn *et al.*, 1996; Saunders & Scheiner-Bobis, 2004).

The results presented here showed, for the first time, that the ET<sub>A</sub> receptor blockade prevents the chronic actions of ouabain on the contractile response of phenylephrine in rat aortae. Here and in previous studies (Rossoni *et al.*, 2002a, b), we have reported that in aorta from ouabain hypertensive rats, the enhanced negative endothelial modulation on the contractile responses to phenylephrine is probably mediated by an increase in eNOS expression and consequent increase of synthesis and NO release. ET<sub>A</sub> receptor blockade completely prevented the increased expression of eNOS and enhanced negative modulatory action of the endothelium caused by treatment with ouabain. The effects of both endothelium removal and NOS inhibition on the actions of phenylephrine in aortic rings from rats treated with Ouabain plus BMS182874 were similar to those obtained in arteries from rats treated with Vehicle plus BMS182874. These results suggest a link between ET-1-activated ET<sub>A</sub> receptors and the increased negative modulatory actions of the endothelium observed in aortic rings from ouabain-treated rats.

To further evaluate the vascular endothelin system in aortae from ouabain-treated rats, we investigated gene expression of pre-pro-ET-1 and its receptors. The expression of mRNAs for pre-pro-ET-1 and ET<sub>A</sub> receptors were increased in aortic rings from ouabain-treated rats. Interestingly, in brain areas of rats associated with cardiovascular regulation, Di Filippo *et al.* (2003) observed about a three-fold increase in ET-1 content and a decrease in the expression of mRNA for ET<sub>A</sub> receptors following treatment with ouabain. Moreover, it was demonstrated that ouabain stimulates ET-1 release and gene expression in endothelial cells (Yamada *et al.*, 1990; Saunders



**Figure 6** Upper panel: Representative blot for eNOS protein expression in aortic arteries from vehicle (Veh) and ouabain-treated (Oua) (a); or vehicle losartan-treated (Veh Los) and Ouabain plus losartan-treated rats (Oua Los) (b); or vehicle BMS184874-treated rats (Veh BMS) and Ouabain plus BMS184874-treated rats (Oua BMS) (c). The first lane shows the positive control (C+, human endothelial cells) for eNOS. Lower panel: Densitometric analysis of the Western blot for eNOS protein expression. Results (means  $\pm$  s.e.m.) are expressed as the ratio between the signal for the eNOS protein and the signal for  $\alpha$ -actin. Unpaired *t*-test, \**P* < 0.05 vs vehicle. Number of animals used is indicated within parentheses.

& Scheiner-Bobis, 2004). The mechanisms by which ouabain induces gene expression and release of ET-1 include both genomic and nongenomic effects that are independent of its ability to inhibit the sodium pump (Saunders & Scheiner-Bobis, 2004).

ET-1 effects are mediated by activation of both ET<sub>A</sub> and ET<sub>B</sub> receptors (Masaki, 2004). In the vascular wall, ET<sub>A</sub> receptors are predominantly expressed in smooth muscle cells, whereas the ET<sub>B</sub> subtype is expressed in both endothelial and smooth muscle cells (Masaki, 2004). The activation of either smooth muscle receptor produces vasoconstriction. In contrast, the activation of ET<sub>B</sub> receptors present on the endothelium produces vasodilation, an effect mediated mainly by synthesis and release of NO (Warner *et al.*, 1989). However, there is evidence suggesting that activation of ET<sub>A</sub> receptors also stimulates NO release. Marsen *et al.* (1999) showed that ET-1 acting on ET<sub>A</sub> receptors induces eNOS gene expression via tyrosine kinase- and protein kinase-dependent pathways. We observed an increase in eNOS protein expression in aortae from ouabain-treated rats that was prevented by BMS182874 treatment. These results suggest that increased expression of mRNA for pre-pro-ET-1 and ET<sub>A</sub> receptors observed in rat

aorta after treatment with ouabain might be responsible for the increased negative endothelial modulation of the contractile actions of phenylephrine in this artery following treatment with ouabain.

In conclusion, we have shown that the changes in phenylephrine vascular reactivity observed in aortae from ouabain hypertensive rats are dependent on the activation of ET<sub>A</sub> receptors by ET-1 and independent of the elevated arterial blood pressure or the renin-angiotensin system. Blockade of ET<sub>A</sub> receptors with BMS182874 prevented both the hypertension and the negative endothelial modulation of the contractile actions of phenylephrine observed after treatment with ouabain. Our results also suggest that chronic treatment with ouabain in rats activates a local endothelin system that, in turn, stimulates eNOS protein expression.

We gratefully acknowledge Professor Louis A. Barker for his helpful discussion and suggestions in the preparation of the article. This work was supported by FAPESP, CNPq, DGICYT (SAF 2003 00633) and Banco Santander Central Hispano. Fabiano E. Xavier was supported by CAPES (PDEE-BEX0339/02-4, Brazil) and by FISS (C03/01, Spain).

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(Received July 12, 2004

Revised August 19, 2004

Accepted September 2, 2004)