*Am J Physiol Heart Circ Physiol* 283: H2110–H2118, 2002.
First published July 11, 2002; 10.1152/ajpheart.00454.2002.

# Ouabain-induced hypertension is accompanied by increases in endothelial vasodilator factors

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[1]*Department of Pharmacology and Therapeutics, Faculty of Medicine, Autonomous University of Madrid, 28029 Madrid, Spain;* [2]*Department of Physiological Sciences, Federal University of Espirito Santo, 29040-090, Brazil; and* [3]*Department of Pharmacology, Louisiania State University Health Sciences Center, New Orleans, Louisiana 70119*

Received 30 May 2002; accepted in final form 9 July 2002

**Rossoni, Luciana V., Mercedes Salaices, Marta Miguel, Ana M. Briones, Louis A. Barker, Dalton V. Vassallo, and María J. Alonso.** Ouabain-induced hypertension is accompanied by increases in endothelial vasodilator factors. *Am J Physiol Heart Circ Physiol* 283: H2110–H2118, 2002. First published July 11, 2002; 10.1152/ajpheart.00454.2002.—The involvement of nitric oxide (NO), prostaglandins, and calcium-dependent potassium channel ($K_{Ca}$) activators on the negative modulation of phenylephrine-induced contractions was evaluated on the isolated aorta and caudal (CAU) artery obtained from rats treated with ouabain for 5 wk to induce hypertension. In ouabain-treated rats, the reactivity to phenylephrine was reduced in the endothelium-intact aorta but not the CAU segments. Endothelial modulation of phenylephrine contraction, as demonstrated by endothelium removal, NO synthase (NOS) inhibition with $N^{\omega}$-nitro-L-arginine methyl ester and aminoguanidine, as well as $K_{Ca}$ inhibition with tetraethylammonium, was more pronounced in segments from ouabain-treated animals, and here greater effects were seen in the aorta than in CAU. An increased expression of endothelial NOS and neuronal NOS was seen in the aorta after ouabain treatment. In CAU, only endothelial NOS was detected and ouabain treatment did not alter its expression. These results suggest that ouabain-induced hypertension is accompanied by increased NO release derived from endothelial NOS and neuronal NOS and increased release of an endothelial hyperpolarizing factor that presumably opens $K_{Ca}$, all of which contribute to the increased negative modulation of the phenylephrine contraction.

nitric oxide; endothelial-dependent hyperpolarizing factor; phenylephrine

THE PLASMA LEVELS of an endogenous circulating $Na^+$-$K^+$-ATPase inhibitor, characterized as ouabain or a closely related compound (17, 36), are increased in several animal models of hypertension (19, 39), as well as in human essential hypertension (20). Several studies have shown that chronic administration of ouabain induces hypertension, an effect that seems to be linked to the inhibition of the $Na^+$-$K^+$-ATPase (10, 22, 23, 45, 54), although sodium pump inhibition seems not to be the exclusive mechanism of the ouabain-hypertensive effect (26, 31, 32, 52). This enzyme is found in most eukaryotic cells and is the main system involved in the maintenance of sodium homeostasis and the membrane potential, essential factors for controlling vascular tone and blood pressure. It has been suggested that alterations in the activity of the sodium pump might be involved in the genesis or maintenance of hypertensive states (3, 33). Additionally, the hypertension induced by ouabain treatment has been associated with actions in the central nervous system that increase sympathetic activity by activation of the central renin-angiotensin system and impair the arterial baroreceptor reflex (22, 23) and associated with actions in the periphery that produce changes in responsiveness to contractile agents (10, 26, 45).

In some isolated vascular preparations, acutely administered ouabain, at nanomolar concentrations, can enhance the actions of phenylephrine (43). Higher micromolar concentrations of ouabain induce contractions by a direct action on the vascular smooth muscle and/or by releasing norepinephrine from the perivascular adrenergic nerve endings (35, 42). In the anesthetized normotensive rat, acutely administered ouabain at doses of 10 and 30 nmol/kg iv can increase arterial pressure, in part, by causing the release of norepinephrine from peripheral nerve endings (2, 44). However, the reactivity of phenylephrine is reduced in some vascular beds following long-term ouabain treatment. We have shown that hypertension induced by chronic administration of ouabain in rats is associated with decreases in the contractile activity of phenylephrine on isolated thoracic aortic and superior mesenteric arteries but not on caudal arteries (45). These alterations were associated with changes in the activity of the sodium pump and the expression of the $\alpha_1$- and $\alpha_2$-isoforms of $Na^+$-$K^+$-ATPase and, in addition, associated with the release of an endothelial factor that negatively modulates vasoconstrictor responses to

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phenylephrine to a greater extent in arteries from ouabain-treated animals than in controls (45). The latter observation is consistent with known acute actions of ouabain that cause the release of endothelial-derived vasodilators such as endothelium-derived hyperpolarizing factor (EDHF), prostacyclin, and nitric oxide (NO) (33, 41, 43, 46, 53), all of which could act to negatively modulate the contractile actions of phenylephrine. However, the nature of the endothelial vasodilator factors involved in the negative modulation of the vasoconstrictor responses in arteries from ouabain-induced hypertensive rats remains unclear.

The aim of the present study was to investigate the endothelial factor(s) involved in the reduction of the vasoconstrictor response to phenylephrine after long-term administration of ouabain. For this, we investigated the role of NO, EDHF, and prostanoids on the modulation of the vasoconstrictor responses induced by phenylephrine in segments from aorta and caudal arteries from control and ouabain-treated rats. In addition, the effect of chronic ouabain treatment on NO synthase (NOS) protein expression was also evaluated. Our results suggest that the release of NO and an EDHF are increased in some vascular beds after long-term ouabain treatment.

## MATERIALS AND METHODS

*Animals.* Six-week-old male Wistar rats were obtained from colonies maintained at the Animal Quarters of the Facultad de Medicina of the Universidad Autónoma of Madrid. During treatment, rats were housed at a constant room temperature, humidity, and light cycle (12:12-h light-dark). Rats had free access to tap water and were fed with standard rat chow ad libitum. All experiments comply with the current Spanish and European laws (RD 223/88 Ministerio de Agricultura, Pesca y Alimentación and 609/86).

*Pellet implantation.* With the rats under diethyl ether anesthesia (Panreac; Barcelona, Spain), a small incision was made on the back of the neck, and one controlled time-release pellet (Innovative Research) containing either ouabain (0.5 mg/pellet) or vehicle (placebo) was implanted subcutaneously according to the method described by Huang et al. (21). These pellets are designed to release a constant amount of either ouabain (~25 μg/day) or vehicle for a 60-day period.

*Blood pressure measurements.* Indirect systolic blood pressure was measured once a week for 5 wk by the tail-cuff plethysmographic method (Letica Digital Pressure Meter, LE 50000 and Pressure Cylinder LE 5100). For this, conscious rats were restrained for 5–10 min in a warm and quiet room and conditioned to numerous cuff inflation-deflation cycles by a trained operator. The average values for systolic blood pressure were subsequently obtained from 10 to 15 sequential cuff inflation-deflation cycles.

After 5 wk, the rats were anesthetized with diethyl ether and killed by exsanguination. Thoracic aorta and caudal arteries were then carefully dissected out and cleaned of connective tissue. For reactivity experiments the arteries were divided into segments of 4 mm in length. For analysis of NOS expression, arteries were rapidly frozen in liquid nitrogen and kept at −70°C until the day of analysis.

*Reactivity experiments.* For isometric tension recording, each segment was set up in an organ bath containing 5 ml of Krebs-Henseleit solution (KHS) at 37°C continuously bubbled with a 95% $O_2$-5% $CO_2$ mixture, pH 7.4. Two horizontally arranged stainless steel pins were passed through the lumen of the vascular cylinder. One pin was fixed to the organ bath wall, whereas the other one was vertically connected to a force-displacement transducer (Letica TRI 011) and to a recorder (MacLab/8e ADInstruments; Castle Hill, Australia). Thoracic aorta segments were subjected to a tension of 1.0 g (optimal resting tension), and caudal segments were subjected to a tension of 0.5 g, which was readjusted every 15 min during a 45-min equilibration period before drug administration.

Vessels were initially exposed to 75 mM KCl to check their functional integrity. Afterward, the presence of endothelium was tested by the effect of acetylcholine (10 μM) on arterial segments previously contracted with phenylephrine at a concentration (~0.1 μM for aorta and 1 μM for caudal arteries) that produces close to 50% of the maximum contraction induced by 75 mM KCl. After a washout period of 60 min, concentration-response curves to phenylephrine were constructed by its cumulative addition (1 nM-10 μM for thoracic aorta and 1 nM-100 μM for caudal artery). The effects of the nonspecific NOS inhibitor $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME, 100 μM), the inducible NO synthase (iNOS) inhibitor aminoguanidine (100 μM), the calcium-activated $K^+$ channel ($K_{Ca}$) blocker tetraethylammonium (TEA, 5 mM), and the cyclooxygenase inhibitor indomethacin (10 μM) on the phenylephrine-elicited response were investigated. For this, these drugs were added 30 min before the concentration-response curve to phenylephrine was generated.

To analyze the influence of endothelium on vascular responses, it was mechanically removed in some experiments by rubbing the lumen with a needle. The absence of endothelium was confirmed by the inability of 10 μM acetylcholine to induce relaxation.

*Western blot analysis of NOS protein expression.* Thoracic aorta and caudal arteries were homogenized in ice-cold Tris-EDTA buffer (in mM: 50 Tris, 1.0 EDTA, pH = 7.4). Homogenates (50 μg protein per lane) and prestained molecular SDS-PAGE standards (Bio-Rad; Hercules, CA) were electrophoretically separated on a 7.5% SDS-PAGE and then transferred to polyvinyl difluoride membranes overnight at 4°C by using a Mini Trans-Blot Cell system (Bio-Rad) containing 25 mM Tris, 190 mM glycine, 20% methanol, and 0.05% SDS. Human endothelial cells, mouse macrophages, and rat pituitary were used, respectively, for endothelial NOS (eNOS)-, inducible NOS (iNOS)-, and neuronal NOS (nNOS)-positive controls. The membrane was then blocked for 60 min at room temperature in Tris-buffered solution (10 mM Tris, 100 mM NaCl, and 0.1% Tween 20) with 5% powdered nonfat milk. Then the membrane was incubated for 1 h at room temperature with mouse monoclonal antibodies for iNOS (1:10,000 dilution), eNOS (1:2,500 dilution), or nNOS (1:2,500), all purchased from Transduction Laboratories (Lexington, UK). After washing was completed, the membrane was incubated with a 1:2,000 dilution of antimouse IgG antibody conjugated to horseradish peroxidase (Transduction Laboratories). The membrane was thoroughly washed, and the immunocomplexes were detected by using an enhanced horseradish peroxidase/luminol chemiluminiscence system (ECL Plus, Amersham International; Little Chalfont, UK) and subjected to autoradiography (Hyperfilm ECL, Amersham International). Signals on the immunoblot were quantified with a National Institutes of Health Image V1.56 computer program. The same membrane was used to determine α-actin expression, and the content of the latter was used to correct NOS expression in each sample by means of a monoclonal antibody anti α-actin (1:30,000 dilution, Boehringer Mannheim; Mannheim, Germany).

*Data analysis and statistics.* Vasoconstrictor responses induced by phenylephrine were expressed as a percentage of the tone generated by 75 mM KCl. The maximum response ($E_{max}$ values) and the negative log of phenylephrine concentrations producing 50% of maximum response ($pD_2$ values) were calculated by a nonlinear regression analysis of each individual concentration-response curve by using GraphPad Prism Software (San Diego, CA).

To compare the effect of different drugs on the response to phenylephrine in segments from control or ouabain-treated 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 curve plot by using a computer program (WinNonlin, version 2.0, Pharsight; Cary, NC); the differences were expressed as a percentage of AUC of the corresponding control situation.

For NOS expression, results are expressed as the ratio between signals on the immunoblot corresponding to isoforms of NOS and α-actin. To compare the results for protein expression within the same experiment and with others, we assigned a value of 1 to the ratio in arteries from control rats and used that value to calculate the relative density of other bands from the same gel.

Results are expressed as means ± SE of the number of rats indicated in each case and analyzed using Student's *t*-test for unpaired experiments or two-way ANOVA to compare groups. When ANOVA showed a significant treatment effect, Tukey's post hoc test was used to compare individual means. A probability value of <5% was considered significant.

*Drugs and solutions.* KHS contained (in mM) 115 NaCl, 25 $NaHCO_3$, 4.7 KCl, 1.2 $MgSO_4 \cdot 7H_2O$, 2.5 $CaCl_2$, 1.2 $KH_2PO_4$, 11.1 glucose, and 0.01 $Na_2EDTA$. Drugs used were the following: acetylcholine hydrochloride, phenylephrine hydrochloride, aminoguanidine hemisulfate, L-NAME dihydrochloride, indomethacin, and TEA (Sigma Chemical; St. Louis, MO); and Tween 20, Tris, SDS, and acrylamide (Bio-Rad). Drug solutions were made in bidistilled water except indomethacin, which was dissolved in 1.5 mM $HNaCO_3$. Stock solutions were kept at −20°C, and appropriate dilutions were made on the day of the experiment.

## RESULTS

*Systolic blood pressure.* After 5 wk, ouabain-treated rats showed significant hypertension compared with the control group [systolic blood pressure in mmHg: untreated, 127 ± 1.4 ($n$ = 24) vs. ouabain-treated, 160 ± 2.1 ($n$ = 35); $P < 0.001$]. No differences in body weight gain were observed (untreated: 156 ± 7 g vs. ouabain treated: 169 ± 10 g, $P > 0.05$).

*Vascular responses to phenylephrine with and without endothelium.* The treatment with ouabain for 5 wk reduced the vasoconstrictor responses induced by phenylephrine in the aorta but not in the caudal rings with endothelium (Fig. 1 and Table 1). Meanwhile, the contraction to 75 mM KCl remained unmodified in the two studied vessels [thoracic aorta: 2,316 ± 102 ($n$ = 12) vs. 2,704 ± 132 mg ($n$ = 9) and caudal: 1,898 ± 71 ($n$ = 13) vs. 2,000 ± 72 mg ($n$ = 9), for untreated and ouabain treated rats, respectively; $P > 0.05$]. Endothelium removal enhanced the phenylephrine responses in thoracic aortas from treated and untreated groups (Fig. 1 and Table 1). However, these changes occurred in a greater extent in arteries from the ouabain-treated rats (dAUC values: 56.6 ± 9.6 vs. 198 ± 18.3% of the corresponding control AUC for untreated and ouabain-treated rats, respectively; $P < 0.05$). In the caudal arteries, the damage of endothelium also increased the phenylephrine response in both groups (Fig. 1 and Table 1). Again, with the comparison of dAUC values in rings without endothelium, this increase was greater in caudal arteries from the ouabain-treated group (13.0 ± 6.1 vs. 39.5 ± 10.4% of the corresponding control AUC for untreated and ouabain-treated rats, respectively; $P < 0.05$). The endothelium damage did not change the contraction induced by 75 mM KCl in the two studied vessels from both groups [thoracic aorta: 2,878 ± 210 ($n$ = 9) vs. 3,081 ± 240 mg ($n$ = 7), and caudal: 1,615 ± 174 ($n$ = 6) vs. 1,598 ± 183 mg ($n$ = 6), for untreated and ouabain-treated rats, respectively; $P > 0.05$].

*Effect of L-NAME and aminoguanidine on the vasoconstrictor responses induced by phenylephrine.* The nonspecific NOS inhibitor L-NAME (100 μM) potentiated the $E_{max}$ to phenylephrine in intact thoracic aorta rings from both group of rats (Fig. 2 and Table 1) but increased $pD_2$ only in vessels from ouabain-treated rats (Table 1). This potentiation was higher in arteries from ouabain-treated rats than in arteries from the control group, as shown by the comparison of dAUC

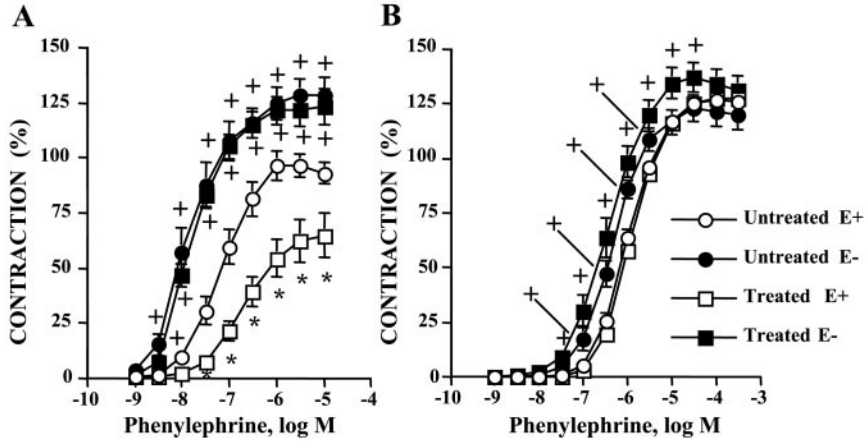


Fig. 1. Concentration-response curve to phenylephrine in intact (E+) and endothelium-denuded (E−) segments of thoracic aorta (*A*) and caudal artery (*B*) of Wistar rats, which received ouabain (treated, $n$ = 6–9) or vehicle (untreated, $n$ = 6–12) subcutaneously for 5 wk. Results (means ± SE) are expressed as a percentage of the response to 75 mM KCl in each case. *$P < 0.01$ vs. untreated rats; +$P < 0.01$ vs. E+.

Table 1. *Effect of endothelium denudation, L-NAME, aminoguanidine, indomethacin, and TEA on $E_{max}$ and $pD_2$ to phenylephrine of thoracic aorta and caudal arteries from rats subcutaneously receiving ouabain (treated) or vehicle (untreated) for 5 wk*

| | Thoracic Aorta | | Caudal Artery | |
|---|---|---|---|---|
| | $E_{max}$ | $pD_2$ | $E_{max}$ | $pD_2$ |
| Control | | | | |
| Untreated | 103 ± 5.9 | 7.15 ± 0.11 (12) | 129 ± 2.8 | 5.98 ± 0.05 (12) |
| Treated | 67.1 ± 10.1* | 6.59 ± 0.10* (9) | 130 ± 1.9 | 5.92 ± 0.04 (9) |
| E− | | | | |
| Untreated | 128 ± 7.5† | 7.80 ± 0.12† (9) | 126 ± 6.4 | 6.32 ± 0.08† (6) |
| Treated | 124 ± 7.0† | 7.79 ± 0.08† (7) | 138 ± 6.8* | 6.43 ± 0.09† (6) |
| L-NAME (100 μM) | | | | |
| Untreated | 147 ± 8.5† | 7.44 ± 0.07 (5) | 131 ± 2.8 | 6.09 ± 0.06 (6) |
| Treated | 144 ± 7.6† | 7.35 ± 0.12† (5) | 127 ± 6.8 | 6.12 ± 0.11 (5) |
| Aminoguanidine (100 μM) | | | | |
| Untreated | 104 ± 5.4 | 6.99 ± 0.09 (6) | | |
| Treated | 111 ± 6.9† | 6.92 ± 0.04† (5) | | |
| Indomethacin (10 μM) | | | | |
| Untreated | 68.8 ± 4.3† | 6.77 ± 0.07† (6) | 119 ± 2.9† | 5.97 ± 0.07 (6) |
| Treated | 42.4 ± 6.8*† | 6.31 ± 0.11* (5) | 120 ± 2.2† | 6.00 ± 0.09 (5) |
| TEA (5 mM) | | | | |
| Untreated | 124 ± 2.4† | 7.23 ± 0.04 (5) | 130 ± 3.3 | 5.83 ± 0.05 (6) |
| Treated | 123 ± 6.7† | 7.06 ± 0.06† (6) | 147 ± 5.5*† | 6.05 ± 0.14 (6) |

Values are means ± SE; number of segments is indicated in parentheses. Ouabain dose was 25 μg/day. E−, endothelial denudation; L-NAME, $N^{\omega}$-nitro-L-arginine methyl ester; TEA, tetraethylammonium; $E_{max}$, maximum response (expressed as a percentage of the response to 75 mM KCl); $pD_2$, negative log of phenylephrine concentrations producing 50% of maximum response. †$P < 0.01$ vs. control; *$P < 0.001$ vs. untreated rats.

values (Fig. 2). L-NAME did not modify the phenylephrine response in caudal arteries from either group (Fig. 2 and Table 1). The iNOS inhibitor aminoguanidine (100 μM) only increased the phenylephrine responses ($E_{max}$ and $pD_2$) in the thoracic aorta from ouabain-treated rats (Fig. 2 and Table 1).

L-NAME, but not aminoguanidine, reduced the relaxation induced by 10 μM ACh in all vessels studied

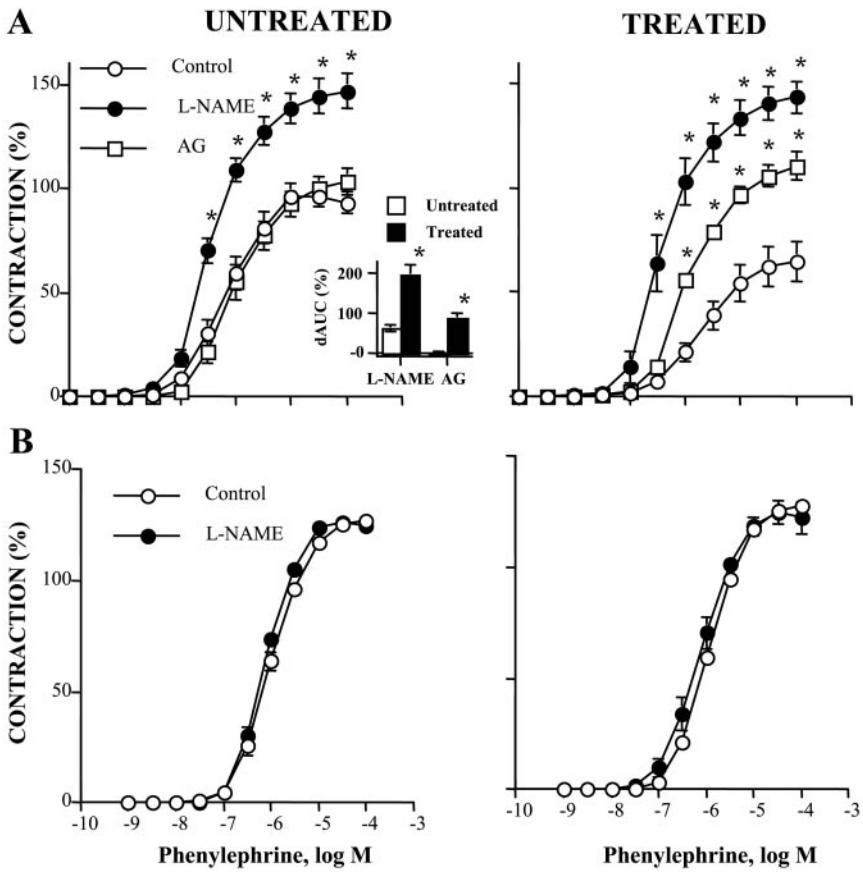


Fig. 2. Effect of 100 μM $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME) and 100 μM aminoguanidine (AG) on concentration-response curve to phenylephrine in segments of thoracic aorta (*A*) and caudal artery (*B*) from Wistar rats, which received ouabain (treated, $n = 5–9$) or vehicle (untreated, $n = 5–12$) subcutaneously for 5 wk. Results (means ± SE) are expressed as a percentage of the response to 75 mM KCl in each case. *$P < 0.01$ vs. control. *Insets*, differences in area under concentration-response curve (dAUC) to phenylephrine in L-NAME or AG and control segments from untreated and ouabain-treated rats; dAUC are expressed as a percentage of the corresponding AUC for control segments. *$P <$ 0.01 vs. untreated.

from both groups (results not shown). Neither L-NAME nor aminoguanidine modified the basal tone of the arteries (results not shown).

*Effect of indomethacin on the vasoconstrictor responses induced by phenylephrine.* The cyclooxygenase inhibitor indomethacin (10 μM) did not modify the basal tone of the two studied vessels from both untreated and ouabain-treated rats. Indomethacin similarly inhibited the response to phenylephrine in the aortic segments from ouabain-treated and untreated rats (Fig. 3). This inhibitor reduced the $E_{max}$ in the aorta from both groups, but it reduced $pD_2$ only in rings from the untreated rats (Table 1). In the caudal arteries, indomethacin slightly reduced the $E_{max}$ to phenylephrine without changes in $pD_2$ in both groups compared with the control curve (Fig. 3 and Table 1). The reduction was similar in segments from both groups, as shown by dAUC values (Fig. 3).

*Effect of TEA on the vasoconstrictor responses induced by phenylephrine.* TEA (5 mM), a $K_{Ca}$ channel blocker, potentiated the $E_{max}$ to phenylephrine in the thoracic aorta rings from both groups (Fig. 3 and Table 1) but increased $pD_2$ only in aortic rings from ouabain-treated rats (Table 1). This potentiation was greater in segments from ouabain-treated than in those from untreated rats, as shown by comparison of dAUC values (Fig. 3). In caudal arteries TEA only potentiated the phenylephrine response in segments from ouabain-treated rats (Fig. 3 and Table 1).

This agent did not modify the basal tone in segments from both ouabain-treated and untreated rats.

*Expression of NOS isoforms.* The eNOS protein expression was increased after ouabain-treatment in the aorta segments, whereas the expression of caudal segments was similar in both groups (Fig. 4). The protein expression of nNOS was only detected in segments from the thoracic aorta; this expression was higher in segments from ouabain-treated rats (Fig. 4). The iNOS isoform was not detected in the vessels from either untreated or ouabain-treated rats (Fig. 4).

## DISCUSSION

As previously demonstrated, long-term treatment with ouabain induces the development of a time-dependent hypertension (10, 21, 23, 26, 32, 45, 54) as well as regional changes in the ouabain-sensitive sodium pump activity and expression of the α-isoforms (45). This hypertension is also associated with a reduction of phenylephrine-induced contractile activity in the thoracic aorta, but not in caudal arteries, and with an increase in the negative endothelial modulation of the actions of phenylephrine in both arteries (45). This negative modulation might constitute a counteregulatory mechanism acting to oppose the increase in blood pressure produced by ouabain. Here, we attempted to determine the nature of the endothelial factor(s) responsible for the increased negative endothelial mod-

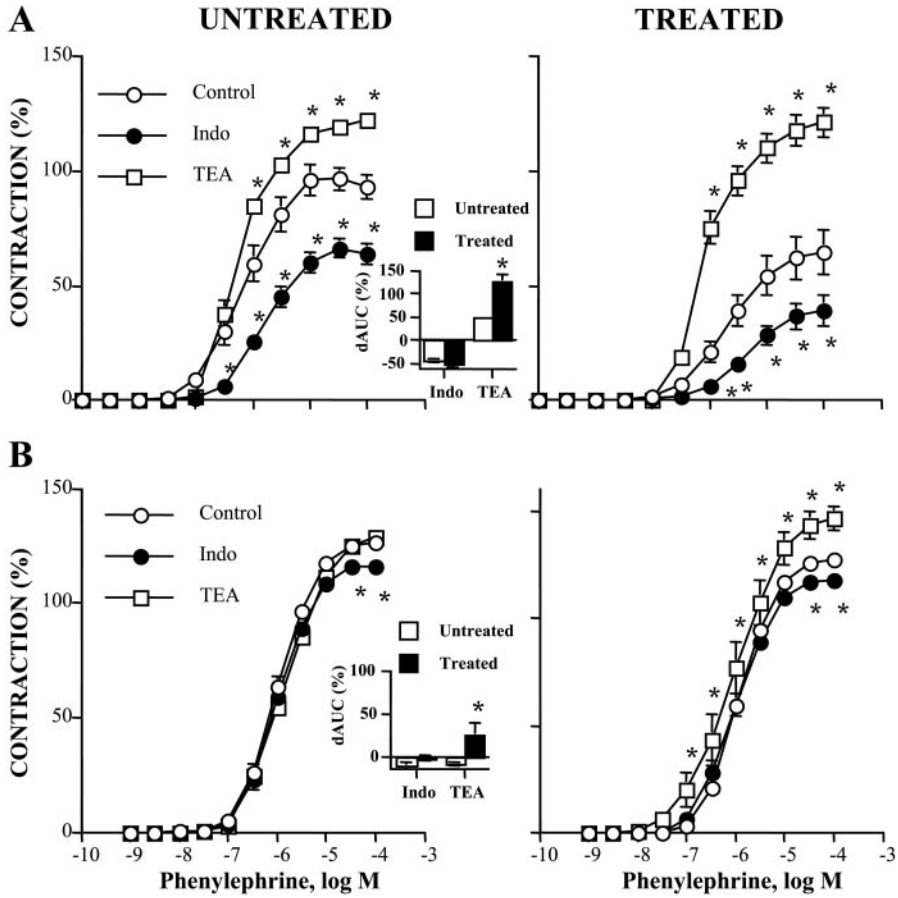


Fig. 3. Effect of 10 μM indomethacin (Indo) and 5 mM tetraethylammonium (TEA) on concentration-response curve to phenylephrine in segments of thoracic aorta (*A*) and caudal artery (*B*) from Wistar rats, which received ouabain (treated, $n = 5–9$) or vehicle (untreated, $n = 5–12$) subcutaneously for 5 wk. Results (means ± SE) are expressed as a percentage of the response to 75 mM KCl in each case. $^*P < 0.01$ vs. control. *Insets*, dAUC to phenylephrine in Indo or TEA and control segments from untreated and ouabain-treated rats; dAUC are expressed as a percentage of the corresponding AUC for control segments. $^*P < 0.01$ vs. untreated.

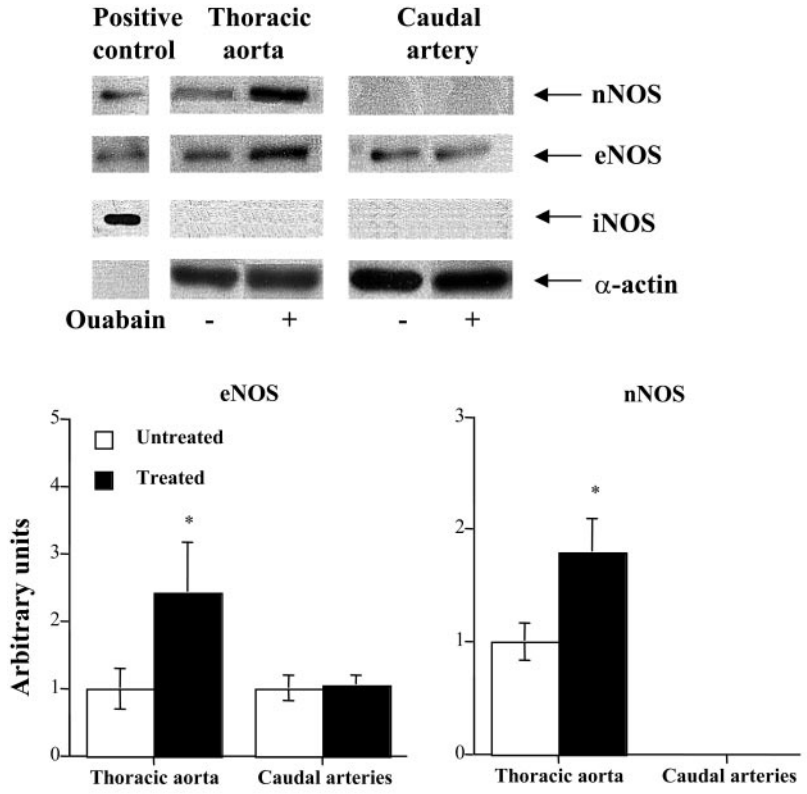


Fig. 4. *Top*: representative Western blot of neuronal (nNOS), endothelial (eNOS), and inducible nitric oxide synthase (iNOS) protein expression in intact segments of thoracic aorta and caudal artery of Wistar rats, which received ouabain (+) or vehicle (−) subcutaneously for 5 wk. *Left lane*, corresponding positive control for each protein (rat pituitary, human endothelial cells, and mouse macrophages, respectively). Expression of α-actin is also shown as a loading control. Arterial homogenates were subjected to SDS-PAGE, followed by immunoblot analysis using anti-nNOS, anti-eNOS, anti-iNOS, and anti-α-actin antibodies. *Bottom*: densitometric analysis of the Western blot of eNOS and nNOS protein expression in intact segments of thoracic aorta and caudal artery of Wistar rats, which received ouabain (treated) or vehicle (untreated) subcutaneously for 5 wk. Results (means ± SE) are expressed as the ratios of the signal of the NOS protein and α-actin of the corresponding segments from ouabain-treated versus those of untreated rats. $^*P < 0.01$ vs. untreated.

ulation of vessels from rats with hypertension induced by chronic treatment with ouabain. Our results suggest that NO and a hyperpolarizing endothelial factor are increased in arteries from ouabain-treated rats. This increase might contribute to the reported reduction of the phenylephrine-induced contraction.

Endothelium plays an important role in the regulation of vasoconstrictor and vasodilator responses elicited by different agonists. It releases vasodilator factors, i.e., NO, EDHF, and prostacyclin, as well as vasoconstrictor factors, i.e., endothelin-1, cyclooxygenase (COX)-derived vasoconstrictor products, and superoxide anion (29, 34). Impairment of endothelium-dependent relaxation has been observed in different vessels from spontaneously hypertensive rats (SHR) and in patients with essential hypertension (34). However, there might be a compensatory increase in the synthesis of endothelial vasodilator factors in hypertension. In SHR an increase in EDHF and in NO production from both iNOS and constitutive NOS (cNOS) has been described (7–9, 12, 30, 38, 50). In addition, hypertension has been associated with alterations in the endothelial modulation of vasoconstrictor responses, including α-adrenergic responses (14). In hypertension induced by long-term administration of ouabain, no impairment of endothelial-dependent relaxation was found (26, 45). On the other hand, a decreased, no modification or an increased contractile response induced by agonists was demonstrated in rings from ouabain-treated rats (10, 26, 45). These contradictory results would be related with the nature of the agonist and with the vessel studied.

Here and previously (45) we showed that the contractile actions of phenylephrine were reduced in isolated aortic rings but not in caudal artery rings obtained from rats treated with ouabain. However, endothelium removal enhances the reactivity to phenylephrine to a greater extent in both arteries from ouabain-hypertensive rats, suggesting an increase in negative endothelial modulation. The acute sensitization or contractile effects of ouabain in different vascular beds seem to be modulated by unknown bioassayable endothelial factor(s) (33, 37, 41, 43, 46, 53). Moreover, ouabain releases prostacyclin and NO from endothelial cells (37, 53), as well as a relaxing factor that seems to open potassium channels (43). However, NO does not appear to be involved in the vasoconstrictor response induced by acute ouabain (41, 46) or in the sensitization of vasoconstrictor responses induced by nanomolar concentrations of ouabain (43).

As indicated above, the results regarding NO modulation of the effects of acute ouabain are controversial. However, after long-term ouabain treatment, the nonselective inhibition of NOS by L-NAME and endothelium removal enhanced the phenylephrine contraction on aortic rings to a greater extent than they did on aortic rings from control animals, whereas L-NAME did not modify the response in caudal arteries. Additionally, long-term treatment with ouabain was accompanied by an increased expression of eNOS and nNOS in the aorta but not caudal arteries. These results suggest that an increase of NO production modulates the contractile activity of phenylephrine in isolated aortic segments. Alterations in NO synthesis or break-

down might be associated with hypertension (25, 34), although contradictory results have been described. Chou et al. (12) found a reduction of both eNOS expression and activity in the aorta from SHR, whereas Briones et al. (8, 9) did not find any alteration in eNOS expression or cNOS activity in the small mesenteric or cerebral arteries from SHR. On the other hand, NO synthesis has been described to be increased with hypertension, probably as a counteregulatory mechanism activated to compensate for the increased blood pressure. Accordingly, an increase of eNOS activity and expression in SHR has been reported (25, 38, 51).

Ouabain has been described to increase NO production and iNOS expression in smooth muscle cells stimulated by interleukin-1β (24, 40). To analyze whether NO derived from iNOS participates in the reduction of phenylephrine-induced contraction observed in the aorta from ouabain-treated rats, the effect of a putative selective iNOS inhibitor, aminoguanidine, was determined. Aminoguanidine, at the concentration used here, did not modify the endothelial-dependent relaxation to acetylcholine in vessels from either untreated or treated rats, suggesting that there was no appreciable inhibition of eNOS. However, aminoguanidine enhanced the contraction to phenylephrine exclusively in segments from ouabain-treated rats. This increase was smaller than the one induced by L-NAME. These results might suggest a potential involvement of NO derived from iNOS in the reduction of the phenylephrine response observed in aortic segments from hypertensive rats. However, there was no measurable expression of this isoform of NOS in arteries from either control or ouabain-treated of rats. Boer et al. (5) showed that aminoguanidine is only about nine times more potent at iNOS than eNOS and almost equipotent at iNOS and nNOS. A recent report has shown that the effect of aminoguanidine in endotoxic shock is most likely due to the inhibition of the nNOS (16). In addition, this isoform has been shown to be increased in arteries from SHR (7, 9). In the present study, we found that the expression of nNOS was measurable in segments of thoracic aorta but not in those of caudal arteries and that this was increased in segments of thoracic aorta from ouabain-treated rats. These results allow us to suggest that NO from nNOS could be, at least partially, involved in the decrease of phenylephrine-induced contraction observed in aorta from ouabain-induced hypertensive rats.

In caudal artery segments, despite the observation of no alteration in phenylephrine response after ouabain treatment, the negative endothelial modulation was also increased. However, L-NAME did not significantly modify the phenylephrine-induced response in segments from ouabain-treated or untreated rats. This suggests a lack of modulation of $\alpha_1$-adrenoceptor response by NO in this vessel, as reported by Tabernero et al. (49). In addition to the functional results, the expression of eNOS protein was similar in segments from both groups. These findings suggest that long-term treatment with ouabain did not modify the endothelial production of NO from eNOS in caudal arteries.

Another endothelial-derived vasodilator that might be involved in the negative endothelial modulation of arteries from ouabain-induced hypertensive rats is prostacyclin. Results from Nagakawa et al. (37) support this idea showing that ouabain induced prostacyclin release from bovine endothelial cells. Moreover, other reports showed that the cyclooxygenase inhibitor indomethacin was unable to alter the acute ouabain vascular effects (41, 46). In addition, it has been shown that hypertension modifies the role of cyclooxygenase-derived products in vasodilator and vasoconstrictor responses (11, 13). In the present study, indomethacin induced a significant reduction of the responses to phenylephrine on thoracic aorta segments and a slight decrease of the maximal response in caudal artery segments from both groups. Rather than support the involvement of a vasodilator prostanoid in the endothelial modulation of the phenylephrine-induced contraction, these results suggest the involvement of vasoconstrictor prostanoids in the contraction to phenylephrine, in agreement with findings of other investigators (27, 48). The inhibitory effect observed was similar in segments from normotensive and ouabain-induced hypertensive rats, arguing against changes of the role of prostanoids from COX in the response to α-adrenoceptor stimulation.

EDHF is another endothelial factor that may modulate the ouabain effects. EDHF is thought to act by opening $K^+$ channels, being $K_{Ca}$ channels frequently involved (1), and/or by stimulating smooth muscle $Na^+$-$K^+$-ATPase (15). Rossoni et al. (43) found that nanomolar concentrations of ouabain induce the release of an endothelium-derived relaxing factor that seems to open $K_{Ca}$ channels. In addition, increased EDHF production has been described in different hypertension models, probably to compensate for the vascular tone increase (30, 50). The $K_{Ca}$ channel blocker TEA potentiated the response to phenylephrine more strongly in segments of aorta from ouabain-treated than untreated rats. This suggest an increased production of a factor that probably opens $K_{Ca}$ channels and would elicit hyperpolarization in segments from ouabain-hypertensive animals. This factor would contribute to the decrease of phenylephrine-induced contraction observed in these vessels. On the other hand, it has been suggested that NO would induce hyperpolarization of smooth muscle cells directly or via cGMP through the opening of $K_{Ca}$ channels (6, 28). This allows us to speculate that the effect of TEA in thoracic aorta segments from hypertensive animals could be, at least partially, due to the hyperpolarizing component from the NO mentioned above.

In segments from the caudal artery, the enhanced actions of TEA were only observed in ouabain-treated rats. This indicates that in the caudal artery from these animals a hyperpolarizing factor is involved in the increased negative endothelial modulation of phenylephrine-induced contraction. This result obtained in the caudal rings from chronic ouabain-treated rats is similar to that obtained for Rossoni et al. (43) in the tail vascular bed after acute administration of ouabain.

Because the response to phenylephrine remained unaltered in caudal arteries after ouabain treatment, some additional factor would have to be increased to compensate for the probably enhanced $K_{Ca}$ activator production.

Results obtained in this study showed, once more, that chronic treatment with ouabain induces hypertension. This kind of hypertension is associated with changes in the activity of the ouabain-sensitive sodium pump and of the expression of the $Na^+$-$K^+$-ATPase isoforms, as well as with regional changes of phenylephrine-induced contractions (45). Results also suggest that in this model for hypertension there is an increase of the negative endothelial modulation of the contractile response to α-adrenergic stimulation. We suggested that in the thoracic aorta there is an increased activity and expression of the sodium pump (45), which could cause hyperpolarization of the vascular smooth muscle and reduce the intracellular calcium concentration by the activation of the $Na^+/Ca^{2+}$ exchanger (3, 4). This increased together with an enhancement of NO production by the activation of eNOS or/and nNOS and the hyperpolarization mediated by the calcium-activated potassium channels reported in this study could explain the reduction of the contractile response to phenylephrine. In addition, NO would also activate the sodium pump, as previously reported (18, 47), enhancing the mechanisms that reduce the contractile response to phenylephrine. Even though, in the caudal artery we previously described an inhibition of the activity and expression of the sodium pump (45). This might reduce the activity of the $Na^+/Ca^{2+}$ exchanger increasing intracellular calcium and consequently smooth muscle contraction. However, this mechanism is counteracted by an increase of negative endothelial modulation via the increment of the hyperpolarization produced by the activation of $K_{Ca}$ channels, without the participation of NO or prostacyclin. The final result could be the maintenance of the contractile response to phenylephrine, as described here.

In conclusion, our results suggest that the increase of negative endothelial modulation on phenylephrine-induced contractions in segments of the thoracic aorta from ouabain-induced hypertensive rats could be due to an increase in endothelial production of both NO, via eNOS and/or nNOS activation, and a hyperpolarizing factor that probably opens $K_{Ca}$ channels. Endothelial hyperpolarizing factor but not NO seems to be increased in segments of caudal artery from hypertensive ouabain-treated rats. In both vessels prostacyclin seems not to be involved in the negative endothelial modulation from hypertensive rats. These increased endothelial factors seem to constitute a counteregulatory mechanism against the elevated blood pressure observed in these animals.

We are grateful to Dr. M. C. Fernández-Criado for the care of animals, A. Lores for skillful technical assistance, and C. F. Warren for linguistic assistance.

This study has been supported by grants from Dirección General de Investigación Científica y Técnica (BX2000 0153) and Conselho Nacional de Pesquisa Brazil (200380/99-0).

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