British Journal of Pharmacology (2002) 135, 771–781

www.nature.com/bjp

Alterations in phenylephrine-induced contractions and the vascular expression of Na^+,K^+-ATPase in ouabain-induced hypertension

[1,2]Luciana V. Rossoni, [1]Mercedes Salaices, [1]Jesús Marín, [2]Dalton V. Vassallo & *,[1]María J. Alonso

[1]Department of Pharmacology and Therapeutics, Facultad de Medicina, Universidad Autónoma de Madrid, Spain and [2]Department of Physiological Sciences, Federal University of Espirito Santo, Vitoria, ES, Brazil

1 Hypertension development, phenylephrine-induced contraction and Na^+,K^+-ATPase functional activity and protein expression in aorta (AO), tail (TA) and superior mesenteric (SMA) arteries from ouabain- (25 μg day^{-1}, s.c., 5 weeks) and vehicle-treated rats were evaluated.

2 Ouabain treatment increased systolic blood pressure (127 ± 1 *vs* 160 ± 2 mmHg, $n=24$, 35; $P<0.001$) while the maximum response to phenylephrine was reduced ($P<0.01$) in AO (102.8 ± 3.9 *vs* 67.1 ± 10.1% of KCl response, $n=12$, 9) and SMA (82.5 ± 7.5 *vs* 52.2 ± 5.8%, $n=12$, 9).

3 Endothelium removal potentiated the phenylephrine response to a greater extent in segments from ouabain-treated rats. Thus, differences of area under the concentration-response curves (dAUC) in endothelium-denuded and intact segments for control and ouabain-treated rats were, respectively: AO, 56.6 ± 9.6 *vs* 198.3 ± 18.3 ($n=9$, 7); SMA, 85.5 ± 15.4 *vs* 165.4 ± 24.8 ($n=6$, 6); TA, 13.0 ± 6.1 *vs* 39.5 ± 10.4% of the corresponding control AUC ($n=6$, 6); $P<0.05$.

4 The relaxation to KCl (1–10 mM) was similar in segments from both groups. Compared to controls, the inhibition of 0.1 mM ouabain on KCl relaxation was greater in AO (dAUC: 64.8 ± 4.6 *vs* 84.0 ± 5.1%, $n=11$, 14; $P<0.05$), similar in SMA (dAUC: 39.1 ± 3.9 *vs* 43.3 ± 7.8%, $n=6$, 7; $P>0.05$) and smaller in TA (dAUC: 62.1 ± 5.5 *vs* 41.4 ± 8.2%, $n=12$, 13; $P<0.05$) in ouabain-treated rats.

5 Protein expression of both α_1 and α_2 isoforms of Na^+,K^+-ATPase was augmented in AO, unmodified in SMA and reduced in TA from ouabain-treated rats.

6 These results suggest that chronic administration of ouabain induces hypertension and regional vascular alterations, the latter possibly as a consequence of the hypertension.
British Journal of Pharmacology (2002) **135,** 771–781

Keywords: Ouabain; hypertension; Na^+,K^+-ATPase; α-isoforms; phenylephrine

Abbreviations: dAUC, difference of area under the concentration-response curves; EDHF, endothelium-derived hyperpolarizing factor; EDTA, ethylenediamine-tetraacetic acid; Emax, maximum response; KHS, Krebs Henseleit solution; NO, nitric oxide; PAGE, polyacrylamide gel; pD_2, negative logarithm of concentrations producing 50% of maximum response; SBP, systolic blood pressure; SDS, sodium lauryl sulphate; SHR, spontaneously hypertensive rats

Introduction

Na^+,K^+-ATPase, the biochemical expression of the electrogenic Na^+ pump, is responsible for the maintenance of cellular membrane potential and can contribute to the regulation of tone and contractility in the vasculature (Blaustein, 1993). The existence of an endogenous circulating Na^+,K^+-ATPase inhibitor in humans and other mammals has been shown (Buckalew & Haddy, 1989). This inhibitor was characterized as ouabain or an isomer of ouabain (Mathews *et al*., 1991), and is found in plasma, brain and adrenal cortex (Ludens *et al*., 1992; Huang & Leenen, 1996). Elevated circulating levels of endogenous ouabain have been demonstrated in humans with essential hypertension (Hamlyn *et al*., 1982) and congestive heart failure (Gottlieb *et al*., 1992) as well as in several animal models of hypertension (Overbeck *et al*., 1976; Hamlyn *et al*., 1996). Ouabain seems to contribute to the regulation of blood pressure and is associated with the development and maintenance of hypertension by central and peripheral mechanisms (Blaustein, 1993; Huang & Leenen, 1996).

Regarding peripheral mechanisms, ouabain in the nanomolar range potentiates agonist-induced contractions in isolated vessel preparations (Rossoni *et al*., 1999). At micromolar concentrations, ouabain induces contraction (Marín *et al*., 1988) by a direct action on the vascular smooth muscle (myogenic component), or by the release of norepinephrine from the perivascular adrenergic nerve endings (neurogenic component) (Marín *et al*., 1988; Rodríguez-Mañas *et al*., 1992). In addition, this glycoside can trigger the release of endothelium-dependent relaxing factors such as endothelium-derived hyperpolarizing factor (EDHF) or nitric oxide (NO), counteracting the vasoconstriction and the sensitization of the vascular smooth muscle induced by ouabain (Xie *et al*., 1993; Ponte *et al*., 1996; Rossoni *et al*., 1999). Moreover, unknown endothelial factors have been suggested to possibly stimulate the sodium pump and partially protect against the inhibition of Na^+,K^+-ATPase produced by ouabain (see Marín & Redondo, 1999).

*Author for correspondence.

Several reports have shown that the chronic administration of ouabain to Wistar rats induces hypertension (Yuan *et al*., 1993; Manunta *et al*., 1994; 2000; Huang *et al*., 1994; Veerasingham & Leenen, 1999; Huang & Leenen, 1999), although others have not found a hypertensive effect after ouabain treatment (Li *et al*., 1995; Pidgeon *et al*., 1996; Cargnelli *et al*., 2000). Ouabain-induced hypertension has also been associated with an increase in sympathetic activity and impairment of the arterial baroreceptor reflex (Huang & Leenen, 1999). However, whether this altered vascular reactivity participates in ouabain-induced hypertension is still unclear.

Thus, the goal of the present study was to determine if chronic treatment with ouabain induced hypertension in Wistar rats and whether this hypertension was associated with changes in vascular reactivity and in the functional activity and protein expression of Na^+,K^+-ATPase. We have evaluated the effect of chronic treatment with ouabain on: (1) the vascular responses to phenylephrine and acetylcholine; (2) the endothelial modulation of vascular reactivity; (3) the relaxation to K^+ in the absence and the presence of ouabain, as an indicator of the functional activity of the Na^+,K^+-ATPase; and (4) the protein expression of isoforms of Na^+,K^+-ATPase α subunit. Because regional differences in vascular reactivity and endothelial modulation of α-adrenoceptor agonists (Oriowo *et al*., 1987; Tabernero *et al*., 1996) as well as in the Na^+,K^+-ATPase activity (Webb & Bohr, 1980; Myers *et al*., 1987) have been reported, this study was performed in three different vessel preparations: thoracic aorta, superior mesenteric and tail artery. This approach was employed to avoid bias resulting from basing a model characterization on a single vascular bed.

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-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 MAPA and 609/86).

Pellet implantation

Under anaesthesia with diethyl ether (Panreac, Barcelona, Spain), a small incision was made in the back of the neck and one controlled time-release pellet (Innovative Research of America, Florida, U.S.A.) containing either ouabain (0.5 mg pellet^{-1}, $n=35$) or vehicle ($n=24$) was implanted subcutaneously, as described by Huang *et al*. (1994). These pellets are designed to release a constant amount of ouabain (approximately 25 μg day^{-1}) or vehicle for a 60-day period.

Blood pressure measurements

Indirect systolic blood pressure was measured once a week for 5 weeks 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 were 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 weeks, the rats were anaesthetized with diethyl ether and sacrificed by exanguination and the thoracic aorta, superior mesenteric and tail arteries were 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 Na^+,K^+-ATPase 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, 150 μm in diameter, were passed through the lumen of the vascular cylinder. One pin was fixed to the organ bath wall, while the other was vertically connected to a strain gauge for isometric tension recording. The isometric contraction was recorded through a force-displacement transducer (Letica TRI 011, Barcelona, Spain) connected to a recorder (MacLab/ 41 ADInstruments Pty Ltd, Castle Hill, Australia). Thoracic aorta segments were subjected to a tension of 9.8 mN (optimal resting tension), and superior mesenteric and tail segments were subjected to a tension of 4.9 mN. This tension 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. Afterwards, concentration-response curves to acetylcholine (0.01 nM–10 μM) were made in arterial segments previously contracted with phenylephrine at a concentration (approximately 0.1 μM for thoracic aorta and approximately 1 μM for mesenteric and tail arteries) that produced close to 50% of the contraction induced by K^+ (75 mM). After a washout period of 60 min, concentration-response curves to phenylephrine were constructed by its cumulative addition (0.1 nM–0.3 mM).

The functional activity of Na^+,K^+-ATPase was indirectly measured in another set of experiments using the method described by Webb & Bohr (1978); after stabilization in KHS with 4.7 mM K^+, the arteries were incubated in a K^+-free medium for 30 min. Afterwards, the vessels were contracted with phenylephrine to obtain approximately 50% of the contraction induced by K^+ (75 mM); once a plateau was reached, K^+ (1–10 mM) was cumulatively added. To determine the effect of 0.1 mM ouabain on these responses, the curve to K^+ was performed in another group of vessels preincubated for 30 min in a K^+-free medium with ouabain.

In some experiments, the influence of the endothelium on the concentration-response to phenylephrine and to K^+ was determined. In these studies, the endothelium was mechanically removed 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 isoforms of α-subunit of the Na^+,K^+-ATPase

Thoracic aorta, superior mesenteric and tail arteries were homogenized in ice-cold Tris-EDTA buffer (in mM: Tris-50, EDTA-1.0, pH = 7.4). The homogenates were centrifuged at $500 \times g$ for 10 min at 4°C and the protein concentration in the supernatant fraction was measured.

Rat kidney microsomal fractions were used as controls for the α_1 and brain microsomal fractions for the α_2 and α_3 isoforms (Sahin-Erdemli *et al*., 1994). Tissues were homogenized in ice-cold Sucrose-Tris-EDTA buffer (in mM: Tris-50, Sucrose-250, EDTA-1.0, pH = 7.4). Initial centrifugation was at $10{,}000 \times g$ for 10 min at 4°C. The supernatant was centrifuged at $100{,}000 \times g$ for 60 min. The pellet, representing the microsomal fraction, was resuspended in Tris-EDTA buffer and the protein concentration was measured.

The samples (30 μg protein for thoracic aorta, 50 μg protein for superior mesenteric and tail artery per lane), as well as the corresponding controls (7–9 μg protein each for kidney and brain homogenates per lane) and prestained molecular SDS–PAGE standards (Bio-Rad, Laboratories, Hercules, CA, U.S.A.) were electrophoretically separated on a 7.5% SDS–PAGE and then transferred to polyvinyl difluoride membranes overnight at 4°C, using a Mini Trans-Blot Transfer Cell system (Bio-Rad) containing (in mM): Tris-25, glycine-250, methanol-20% and SDS-0.05%. Then, the membrane was blocked for 60 min at room temperature in Tris-buffered solution (in mM: Tris-25, NaCl-137, Tween 20-0.2%, pH 7.5) with 5% powdered non-fat milk. Next, the membrane was incubated for 90 min at room temperature with anti-α_1 rabbit polyclonal IgG (0.1 μg ml^{-1} dilution), anti-α_2 rabbit polyclonal antiserum (1 : 5000 dilution) or anti-α_3 rabbit polyclonal antiserum (0.5 μg ml^{-1} dilution), all purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.). After washing, the membrane was incubated for 90 min with an anti-rabbit IgG antibody conjugated to horseradish peroxidase (1 : 3000 dilution) (Transduction Laboratories, Lexington, U.K.). The membrane was thoroughly washed and the immunocomplexes were detected using an enhanced horseradish peroxidase/luminol chemiluminiscence system (ECL Plus, Amersham International plc, Little Chalfont, U.K.) and then subjected to autoradiography (Hyperfilm ECL, Amersham International plc) for either 1–2 min (α_1) or 5–10 min (α_2 and α_3). Signals on the immunoblot were quantified with a NIH Image V1.56 computer program.

Data analysis and statistics

Vasoconstrictor responses induced by phenylephrine were expressed as a percentage of the tone generated by 75 mM K^+. Relaxation induced by acetylcholine or K^+ was expressed as a percentage of the tone previously obtained with phenylephrine. The maximum response (Emax values) and the negative logarithm of concentrations of phenylephrine or acetylcholine producing 50% of maximum response (pD_2 values), were calculated by a non-linear regression analysis of each individual concentration-response curve using GraphPad Prism Software (San Diego, CA, U.S.A.).

In order to compare the effect of endothelium removal or *in vitro* ouabain on the responses to phenylephrine or KCl 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 using a computer program (WinNonlin v2.0, Pharsight Corp, Cary, NC, U.S.A.); the differences were expressed as a percentage of AUC of the corresponding control situation.

To compare the results for isoforms of the α-subunit of Na^+,K^+-ATPase protein expression within the same experiment and with others, we assigned a value of 1 to either α_1 or α_2 expression in arteries from control rats and used that value to calculate the relative density of other bands from the same gel. When loading, special care was taken to be sure of the amount of sample loaded. Preliminary experiments showed that increasing the loaded protein concentrations gave proportional actin and α_1 signals.

Results are expressed as mean ± s.e.mean of the number of rats indicated in each case. Data were analysed using the Student's *t*-test for unpaired experiments or by one- or two-way analysis of variance (ANOVA) to compare groups. When ANOVA showed a significant treatment effect, Tukey's *post hoc* test was used to compare the different treatment groups. A probability value of less than 5% was considered significant.

Drugs and solutions

KHS contained (mM): NaCl 115, $NaHCO_3$ 25, KCl 4.7, $MgSO_4$. $7H_2O$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, glucose 11.1 and Na_2EDTA 0.01. The K^+ free KHS was prepared by omitting KCl and replacing KH_2PO_4 with NaH_2PO_4. Drugs used were: acetylcholine hydrochloride, ouabain octahydrate and phenylephrine hydrochloride (Sigma Chemical Co., St. Louis, MO, U.S.A.); Tween 20, Tris, SDS and acrylamide (BioRad, Laboratories, Hercules, CA, U.S.A.) and methanol and sucrose (Merck KGaA, Darmstadt, Germany). Drug solutions were made in bidistilled water. Stock solutions were kept at −20°C and appropriate dilutions were made on the day of the experiment.

Results

Systolic blood pressure

Figure 1 shows the evolution of rat systolic blood pressures (SBP) during ouabain (25 μg day^{-1}) or vehicle treatment. A progressive increase of the SBP was observed in the ouabain-treated group from the first week. In the control group (rats treated with vehicle), there was a small increase in SBP after the third week; this rise in SBP was the same that is normally observed in growing rats. At the end of the study, the ouabain-treated rats showed significant hypertension as compared to the control group (control, 127 ± 1, $n = 24$; ouabain 160 ± 2 mmHg, $n = 35$, $P < 0.001$).

No differences in body weight gain were observed (161 ± 8 and 167 ± 4 g, $P > 0.05$, in control and ouabain-treated rats, respectively).

Vascular responses to phenylephrine and acetylcholine

To provide maximal activation for each preparation rings were contracted with 75 mM KCl. This treatment induced

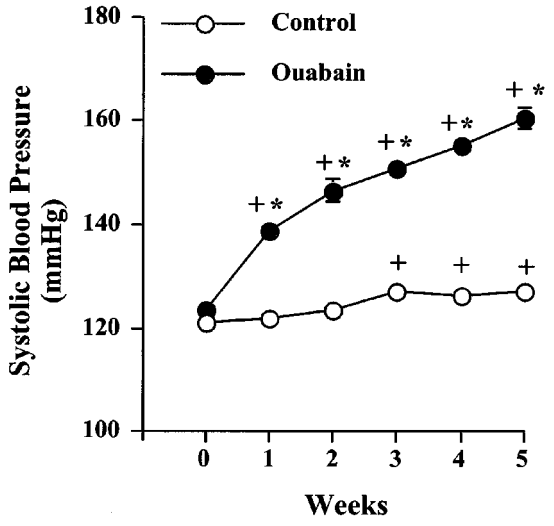

Figure 1 Systolic blood pressure for Wistar rats which received ouabain ($n=35$) or vehicle ($n=24$) subcutaneously for 5 weeks. Results are expressed as mean ± s.e.mean for the number of animals used. $^*P<0.01$ *vs* control rats; $+P<0.05$ *vs* 0 week.

similar contractions in vessels from control and ouabain-treated rats (thoracic aorta: control, 22.7 ± 1.0, $n=12$ *vs* ouabain, 26.5 ± 1.3 mN, $n=9$, $P>0.05$; superior mesenteric artery: control, 12.5 ± 1.3, $n=12$, *vs* ouabain, 13.4 ± 0.8 mN, $n=9$, $P>0.05$; tail artery: control, 18.6 ± 0.7, $n=13$, *vs* ouabain, 19.6 ± 0.7 mN, $n=9$, $P>0.05$).

Treatment with ouabain for 5 weeks reduced the vasoconstrictor responses induced by phenylephrine (0.1 nM – 0.3 mM) in endothelium-intact aorta and mesenteric arteries but did not change this response in tail arteries (Figure 2). In aorta, ouabain treatment reduced both Emax and pD_2 whereas only Emax was reduced in mesenteric arteries (Table 1). In addition, *AUC* to phenylephrine was reduced in aorta (210.9 ± 17.1, $n=12$, *vs* 109.4 ± 17.6 arbitrary units, $n=9$, for control and ouabain-treated rats, respectively, $P<0.001$) and mesenteric arteries (158.4 ± 23.7, $n=12$, *vs* 88.2 ± 11.3 arbitrary units, $n=9$, for control and ouabain-treated rats, respectively, $P<0.05$) but not in tail arteries (253.1 ± 9.0, $n=12$, *vs* 237.5 ± 4.5 arbitrary units, $n=9$, for control and ouabain-treated rats, respectively, $P>0.05$).

The contractions induced by 75 mM KCl were unaltered by endothelium removal (results not shown) in both control ($n=12$, 8, 11 for aorta, mesenteric and tail arteries, respectively) and ouabain-treated ($n=15$, 9, 14 for aorta, mesenteric and tail arteries, respectively) rats; however, the vasoconstrictor response to phenylephrine was potentiated in all studied arteries, in both control and ouabain-treated rats (Figure 3). In aorta and mesenteric arteries from both groups endothelium removal increased both Emax and pD_2 to phenylephrine, while only pD_2 was increased in the tail artery from control and ouabain-treated rats (Table 1). The comparison of dAUC values indicates that after endothelium removal the potentiation of the phenylephrine-induced contraction in arteries from ouabain-treated hypertensive rats was greater than that observed in arteries from control normotensive rats (Figure 3).

The endothelium-dependent relaxation induced by acetylcholine (0.01 nM – 10 μM) was unaltered by ouabain treatment in the three arteries studied (Table 1).

Na^+,K^+-ATPase activity

In intact vessels previously contracted by phenylephrine after exposure to K^+-free medium, the increase of extracellular K^+ concentration (1 – 10 mM) induced a vasodilation that was similar in arteries from control and ouabain-treated rats (Figure 4 and AUC values: aorta, 781.3 ± 19.4, $n=6$, *vs* 790.6 ± 15.4, $n=8$, $P>0.05$; mesenteric, 875.0 ± 6.2, $n=6$, *vs* 862.0 ± 4.2, $n=7$, $P>0.05$; tail artery, 713.5 ± 47.4, $n=6$, *vs* 713.3 ± 56.3 arbitrary units, $n=8$, $P>0.05$, for control and ouabain-treated rats, respectively). After endothelium removal, the relaxation induced by increasing concentrations of K^+ after K^+-free medium exposure was reduced in the three vessels studied from both control and ouabain-treated rats (Figure 4). In these conditions, the K-induced relaxation was smaller in segments from ouabain-treated rats (Figure 4).

Preincubation of intact segments with ouabain (0.1 mM) for 30 min in K^+-free medium induced a slight increase of vascular tone that was similar ($P>0.05$) in arteries from control and ouabain-treated rats (aorta: 6.2 ± 2.8, $n=11$, *vs* 9.9 ± 3.6, $n=14$; mesenteric: 23.3 ± 3.6, $n=6$, *vs* 22.4 ± 7.5, $n=7$; caudal: 7.3 ± 3.4, $n=12$, *vs* 7.3 ± 3.8% of 75 mM KCl response, $n=13$) and reduced the K^+-induced vasodilation (Figure 5). However, long-term ouabain treatment had different actions on the inhibitory effect of *in vitro* ouabain on the K^+-induced relaxation in the three isolated vascular preparations. In mesenteric arteries, this reduction was similar in control and ouabain-treated rats; in contrast, in aorta and tail arteries from ouabain-hypertensive rats this reduction was, respectively, greater and smaller than in those from control rats (Figure 5).

Western blot analysis of Na^+,K^+-ATPase α_1 and α_2 isoform expression

Figure 6 shows the α_1 and α_2 isoforms detected in thoracic aorta, mesenteric and tail arteries. Ouabain treatment increased α_1 and α_2 expression in aorta, reduced it in tail arteries and did not change it in mesenteric arteries (Figure 6). The α_3 isoform was undetected in the three vessels studied, although it was detected in brain microsomal fractions (Figure 6).

Discussion

The results obtained in the present study show that chronic treatment with ouabain induces the development of time-dependent hypertension. This hypertension is associated with a decrease in the phenylephrine-induced contractions, probably due to an increase of the endothelial modulation of this contraction. Moreover, this hypertension is associated with alterations in the ouabain-sensitive Na^+,K^+-ATPase activity and protein expression of α-isoforms. Regional differences on the vasopressor responses to phenylephrine and in the ouabain-sensitive Na^+,K^+-ATPase activity and protein expression resulting from ouabain treatment are found.

Ouabain is an endogenous digitalis compound found in the plasma of several mammals (Overbeck *et al*., 1976; Hamlyn *et al*., 1996); endogenous levels have been reported to be increased in some pathological conditions such as hyperten-

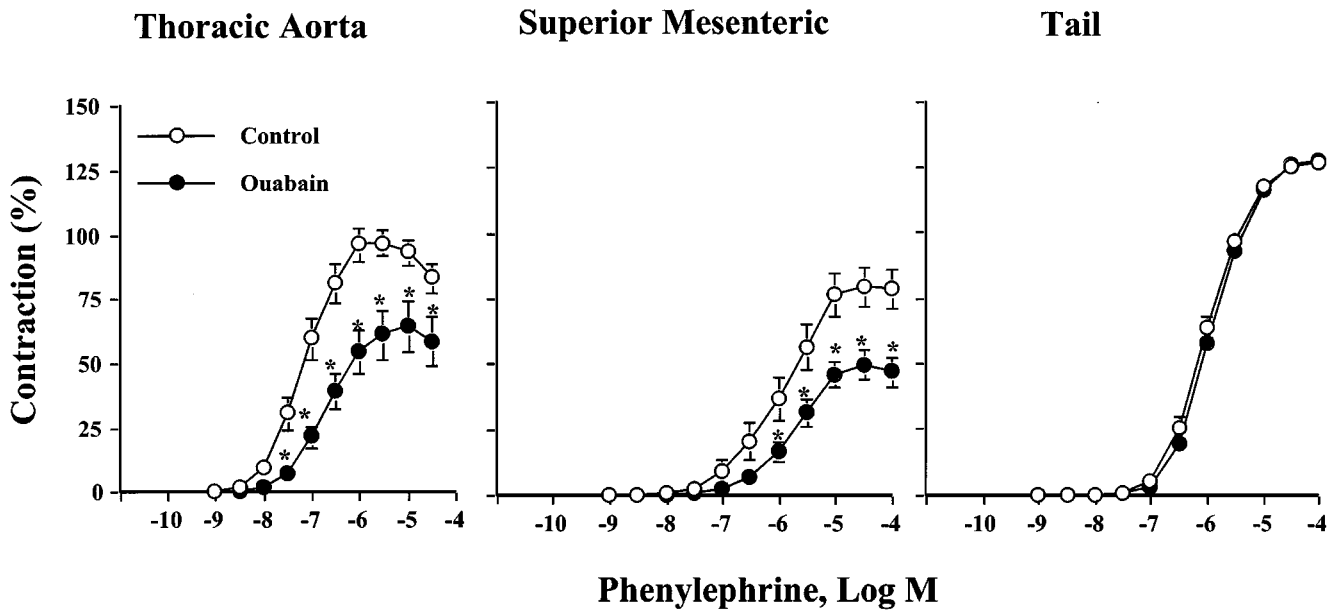

Figure 2 Concentration-response curve to phenylephrine in intact segments from thoracic aorta, superior mesenteric and tail arteries of Wistar rats which received ouabain ($n=9$) or vehicle ($n=12$) subcutaneously for 5 weeks. Results (mean ± s.e.mean) are expressed as a percentage of the response to 75 mM KCl in each case. $^{*}P<0.01$ *vs* control rats.

Table 1 Maximum response (Emax) and sensitivity (pD_2) to phenylephrine (Phenyl) and acetylcholine (ACh) of intact (E+) or denuded (E−) segments from rats subcutaneously receiving ouabain (25 μg day^{-1}) or vehicle for 5 weeks

			Thoracic Aorta		*Superior Mesenteric*		*Tail*	
			Emax	*pD2*	*Emax*	*pD2*	*Emax*	*pD2*
Phenyl	Control	E+	102.8±3.9	7.15±0.11 (12)	82.5±7.5	5.89±0.12 (12)	128.9±3.0	5.99±0.05 (12)
		E−	128.4±7.5^{+}	7.80±0.12^{+}(9)	104.4±6.5^{+}	6.88±0.10^{+}(6)	126.0±6.4	6.32±0.08^{+}(6)
	Ouabain	E+	67.1±10.1*	6.60±0.10* (9)	52.2±5.8*	5.70±0.11 (9)	130.0±1.6	5.89±0.05 (9)
		E−	124.0±7.0^{+}	7.79±0.08^{+}(7)	88.4±6.3^{+}	6.68±0.12^{+}(6)	138.2±6.8*	6.43±0.09^{+}(6)
ACh								
	Control	E+	91.4±1.9	8.10±0.11 (12)	95.1±2.2	7.90±0.12 (12)	74.1±3.6	6.81±0.11 (12)
	Ouabain	E+	92.9±1.6	7.95±0.14 (9)	96.0±1.6	8.08±0.10 (9)	70.7±2.8	7.04±0.10 (9)

Emax values are expressed as a percentage of the response to 75 mM KCl (for phenylephrine) or as a percentage of the previous tone induced by phenylephrine (for acetylcholine). $^{+}P<0.05$ *vs* E+, $^{*}P<0.05$ *vs* control rats. Number of segments is indicated in parentheses.

sion (Hamlyn *et al*., 1982; 1996; Gottlieb *et al*., 1992). Central mechanisms (Huang *et al*., 1994; Veerasingham & Leenen, 1999; Huang & Leenen, 1999) and peripheral mechanisms, such as the increase of myocardial contraction and vascular smooth muscle tone (Marín *et al*., 1988; Blaustein, 1993) as well as increased vascular reactivity to pressor agents (Vassallo *et al*., 1997; Rossoni *et al*., 1999; Davel *et al*., 2000), have all been proposed as mechanisms for ouabain induction of hypertension.

Contributing to the hypothesis that ouabain could be associated with the genesis of hypertension, our results show that peripheral administration of ouabain for 5 weeks induced hypertension in Wistar rats, which was evident from the first week of treatment. There are several reports showing that chronic treatment with ouabain induces hypertension in rats (Yuan *et al*., 1993; Manunta *et al*., 1994; 2000; Huang *et al*., 1994; Veerasingham & Leenen, 1999; Huang & Leenen, 1999; Kimura *et al*., 2000), although other studies found a lack of a hypertensive effect after ouabain administration in rats (Li *et al*., 1995; Cargnelli *et al*., 2000) and sheep (Pidgeon *et al*., 1996). These contradictory results could be due to differences in the duration, dosage, route of ouabain administration or differences in rat strains, or age at the beginning of the treatment.

To determine the possible participation of the vascular component in the ouabain-induced hypertension, we investigated the contraction induced by phenylephrine in rings from thoracic aorta, superior mesenteric and tail artery. There was a gradient in the effect of ouabain on the phenylephrine response in rings from the three vessels studied. Thus, in aorta, both Emax and sensitivity were reduced, in mesenteric artery only Emax was reduced, while in the tail artery no differences in either parameter were found. Nevertheless, the contractile response to high potassium remained unmodified after ouabain treatment in the three studied vessels; this suggests that the treatment does not impair the ability of smooth muscle cells to contract. The results from aortic rings agree with those of Cargnelli *et al*. (2000), despite the absence of any increase in systolic blood pressure. However, another recent study has proposed that chronic treatment with

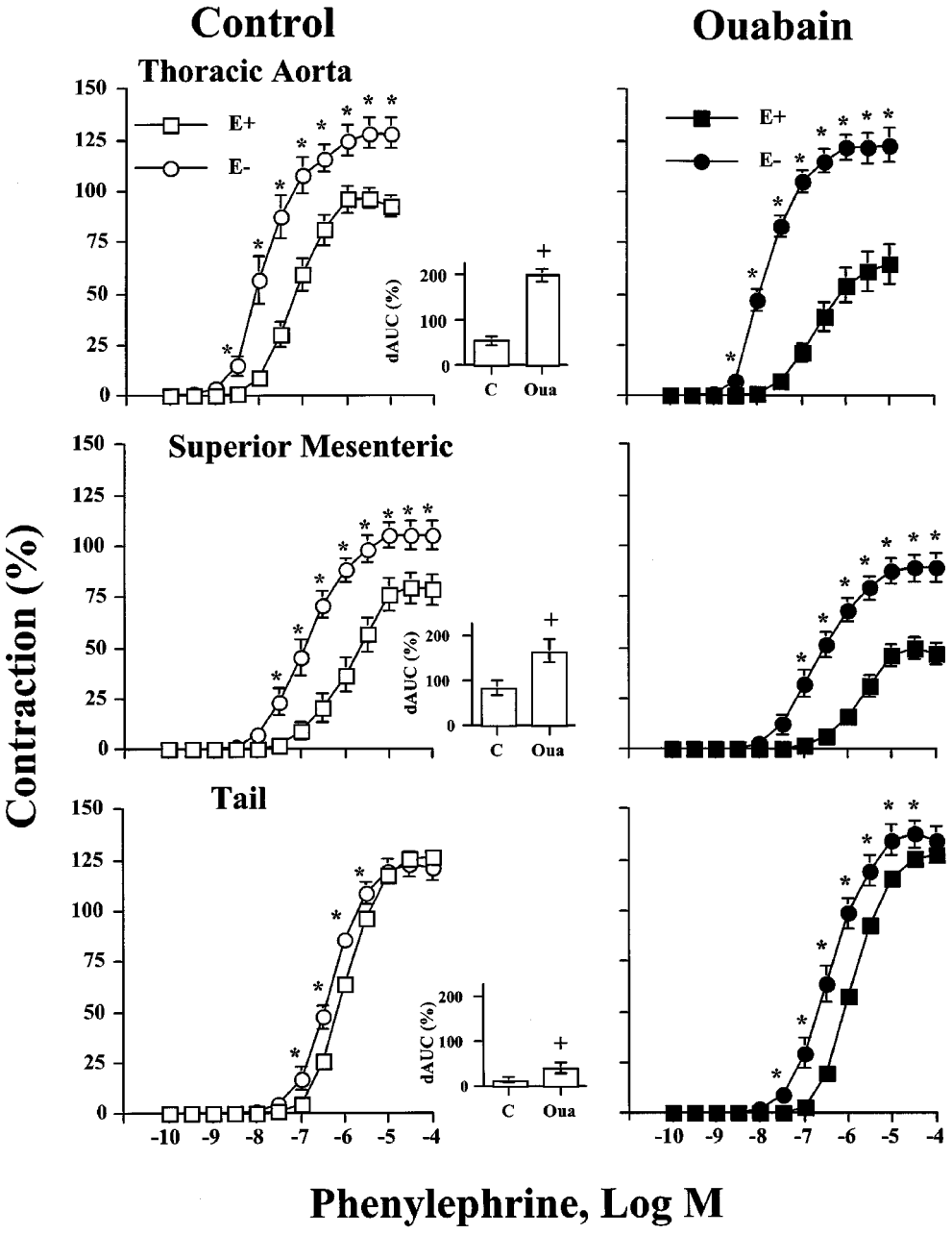

Figure 3 Concentration-response curve to phenylephrine in intact (E+) and endothelium denuded (E−) segments from thoracic aorta, superior mesenteric and tail arteries of Wistar rats which received ouabain ($n=6-9$) or vehicle ($n=6-12$) subcutaneously for 5 weeks. Results (mean ± s.e.mean) are expressed as a percentage of the response to 75 mM KCl in each case. $*P<0.01$ *vs* E+. Inserts show differences in area under the concentration-response curve (dAUC) to phenylephrine in endothelium-denuded and intact segments from control (C) and ouabain-treated (Oua) rats; dAUC are expressed as a percentage of the corresponding AUC for intact segments. $+P<0.01$ *vs* control.

ouabain induces hypertension and increases the contraction evoked by potassium and phenylephrine in renal arteries (Kimura *et al*., 2000).

It is usually assumed in different animal models of hypertension as well as in essential human hypertension, there is some impairment of endothelium-dependent vasodilator responses, although enhancement or no modification have also been described (Marín & Rodríguez-Martínez, 1997). After ouabain treatment, we observed no change of the endothelium-dependent relaxation to acetylcholine in any of the vessels studied, as was also reported for renal arteries (Kimura *et al*., 2000). Thus, our results cannot support an association between these effects and the observed increase in systolic blood pressure, although they do suggest a compensatory mechanism.

One hypothesis to explain the reduced response to phenylephrine in arteries from ouabain-treated rats is that this treatment increases the production of endothelium-derived relaxing factor(s). It is known that negative endothelial modulation of vasoconstrictor responses, includ-

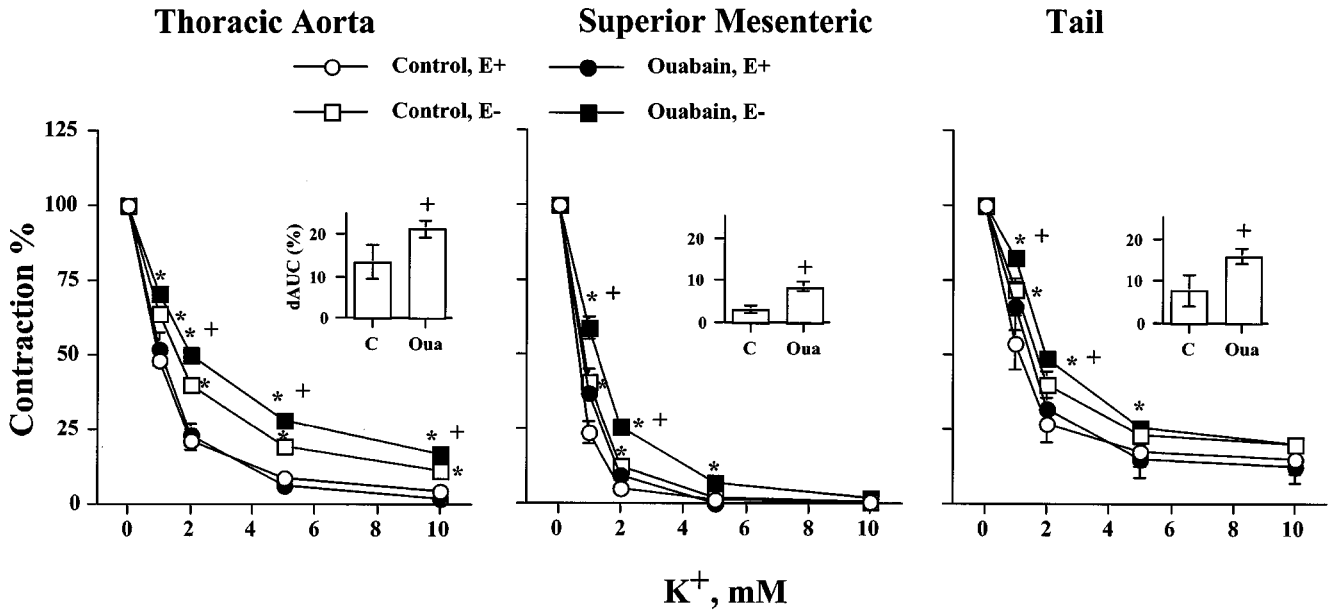

Figure 4 Concentration-response curve to potassium in intact (E+) and endothelium denuded (E−) segments from thoracic aorta, superior mesenteric and tail arteries of Wistar rats which received ouabain ($n=7-15$) or vehicle ($n=6-12$) subcutaneously for 5 weeks, previously incubated in a K^+-free medium and contracted with phenylephrine. Results (mean ± s.e.mean) are expressed as a percentage of the response to phenylephrine in each case. Inserts show differences in area under the concentration response curve (dAUC) to potassium in endothelium-denuded and intact segments from control (C) and ouabain-treated (Oua) rats; dAUC are expressed as a percentage of the corresponding AUC for intact segments $*P<0.05$ *vs* E+; $+P<0.01$ *vs* control rats.

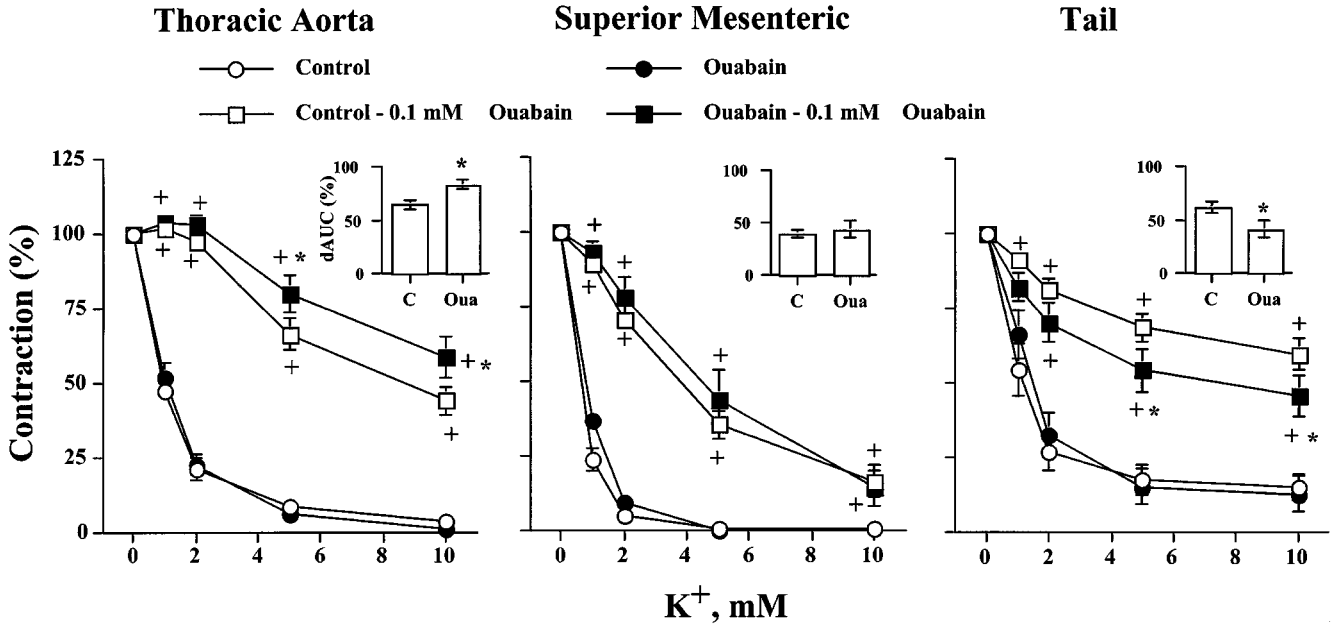

Figure 5 Effect of 0.1 mM ouabain on potassium-induced relaxations in intact segments from thoracic aorta, superior mesenteric and tail arteries of Wistar rats which received ouabain ($n=7-14$) or vehicle ($n=6-12$) subcutaneously for 5 weeks, previously incubated in a K^+-free medium and contracted with phenylephrine. Results (mean ± s.e.mean) are expressed as a percentage of the response to phenylephrine in each case. Inserts show differences in area under the concentration-response curve (dAUC) to potassium in segments in the absence and in the presence of *in vitro* ouabain, from control (C) and ouabain-treated (Oua) rats; dAUC are expressed as a percentage of the corresponding AUC in the absence of ouabain. $*P<0.05$ *vs* control rats; $+P<0.01$ *vs* response in the absence of ouabain.

ing α-adrenergic responses (Carrier & White, 1985), is increased in spontaneously hypertensive rats (SHR) (Arribas *et al*., 1994), although a decrease of negative endothelial modulation in SHR has also been described (Dohi *et al*., 1996). On the other hand, Rossoni *et al*. (1999) have recently suggested that nanomolar concentrations of ouabain induce

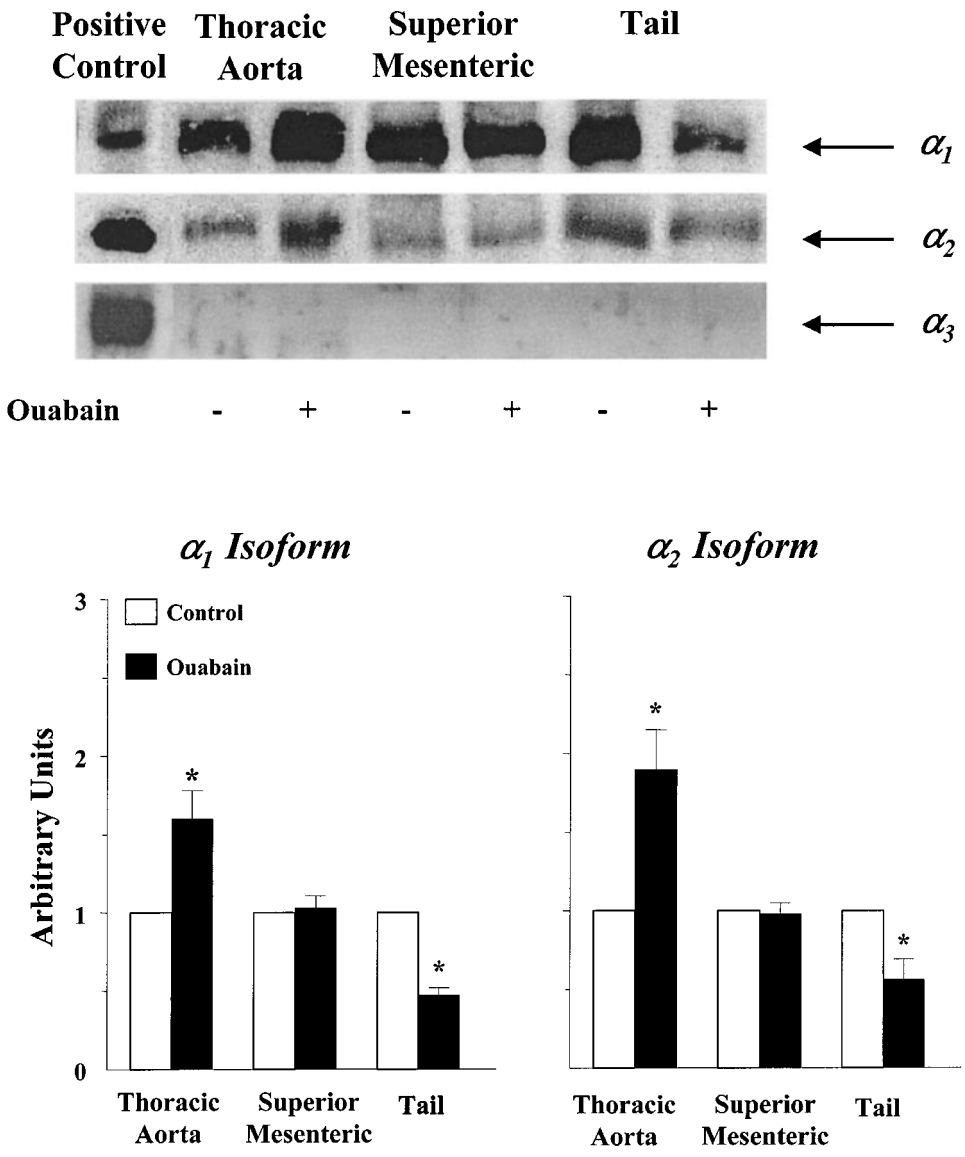

Figure 6 Upper panel. Representative Western blot for α_1, α_2 and α_3 isoforms protein expression in intact segments from thoracic aorta, superior mesenteric and tail artery of Wistar rats which received ouabain (+) or vehicle (−) subcutaneously for 5 weeks. The first lane shows the corresponding positive control for each protein (kidney for α_1 and brain for α_2 and α_3). Lower panel. Densitometric analysis of the Western blot of α_1 and α_2 isoform protein expression in intact segments from thoracic aorta, superior mesenteric and tail arteries of Wistar rats subcutaneously receiving ouabain ($n=7-8$) or vehicle ($n=5-6$) for 5 weeks. Results (mean ± s.e.mean) are expressed as the ratio of the signal of the α_1 or α_2 protein of the corresponding segments from ouabain-treated *versus* that of control rats. *$P<0.05$ *vs* Control.

the release of an endothelium-derived relaxing factor that seems to open potassium channels. Other reports have also suggested that ouabain induces the release of nitric oxide in cultured endothelial cells (Xie *et al*., 1993) or that this compound stimulates inducible NO synthase expression in vascular smooth muscle cells (Pacheco *et al*., 2000). In endothelium-denuded segments from aorta and superior mesenteric artery there was an increase in the vasoconstrictor response to phenylephrine, and this increase was stronger in segments from ouabain-treated rats. These results contrast with those of Cargnelli *et al*. (2000) who found that endothelium removal reduced the contractile response to phenylephrine in aorta from ouabain-treated rats. Our results suggest that after ouabain treatment there is an increase in negative endothelial modulation of phenylephrine-induced responses. This phenomenon could explain the hyporesponsiveness to phenylephrine-induced contraction that we observed. Nevertheless, additional experiments are necessary to identify the endothelial factor(s) that might be increased after ouabain treatment.

The increase of the phenylephrine response after endothelial denudation was greater in tail artery segments from ouabain-treated rats, but still much lower than that found in aorta and mesenteric artery. If ouabain induces the release of an endothelial vasodilator factor and the endothelium modulates vasoconstrictor responses to a lesser extent in the tail artery than in other vascular beds, it is likely that the response to α-adrenergic stimulation is less affected by

ouabain treatment. Interestingly, the magnitude of the increase of the phenylephrine-induced contraction after endothelium removal seems to be related to the ability of acetylcholine to induce relaxation, and this may reflect the ability of endothelium to release vasoactive factors. However, as mentioned above, the endothelium-dependent relaxation to acetylcholine was not modified after ouabain treatment. Thus, although ouabain increases endothelial vasodilator factors release in response to phenylephrine, this increase does not occur in the response to acetylcholine, suggesting differences in the mechanisms of endothelial factors release.

As the effects of ouabain are usually explained by its ability to inhibit Na^+,K^+-ATPase, the activity of this pump was measured by a usual procedure described by Webb & Bohr (1978). The addition of K^+ elicited concentration-dependent relaxations that almost fully reversed the tone induced by phenylephrine in a K^+-free medium in the three studied vessels. It has been proposed that endothelial factors could stimulate the Na pump and partially protect it against inhibition by ouabain (see Marín & Redondo, 1999). Our results confirm the existence of a positive endothelial modulation of the Na pump in the three vessels studied. This endothelial modulation was slightly stronger in segments from ouabain-treated rats, supporting the observed higher endothelial modulation found in these arteries. The inhibitory effect of a high concentration of ouabain on the relaxation induced by K^+ was different in intact segments from ouabain-treated and control rats. Ouabain reduced the K^+-elicited relaxation more in thoracic aorta from ouabain-treated rats than in those from control rats. In superior mesenteric arteries, the effect of ouabain was similar, but in the tail artery, it was smaller in ouabain-treated rats than in controls. These results suggest that ouabain-sensitive Na^+,K^+-ATPase activity was increased in aorta, unaltered in mesenteric rings and reduced in tail artery from the ouabain-induced hypertensive rats. Na^+ pump activity has been reported to be reduced in vessels from SHR (Ponte *et al*., 1996) and renal hypertensive dogs (Overbeck *et al*., 1976), although increased activity has also been reported in SHR (Cox, 1986). Redondo *et al*. (1995) found that the Na^+ pump activity in aortic cultures of smooth muscle from SHR and Wistar Kyoto rats was similar, with no differences in either the number of Na^+ pump sites and/or activity, although the regulation of its activity seems to be significantly different.

The Na^+ pump is composed of two major subunits, α and β, the α subunit being the catalytic component (Blanco & Mercer, 1998; Marín & Redondo, 1999). A third subunit, named γ, has been also reported, but its role, if any, is not well defined (see Blanco & Mercer, 1998; Marín & Redondo, 1999). Four different α isoforms, α_1, α_2, α_3, α_4, of Na^+,K^+-ATPase, with different affinities for digitalics, and expressed in species- and tissue-specific manners, have been identified (Sweadner, 1989; Blanco & Mercer, 1998). Rat vascular tissues have been reported to only express the α_1, α_2 and α_3 isoforms (Sahin-Erdemli *et al*., 1994). Our results showed that, in rat aorta, superior mesenteric and tail arteries only the expression of the α_1 and α_2 isoforms could be detected. Small amounts of the α_3 isoform in vascular smooth muscle and a very low sensitivity of the antibody used could result in cross-reaction with the α_2 isoform, which would explain the lack of α_3 expression detection. Chronic ouabain administration changed the protein expression of the α-subunit isoforms of Na^+,K^+-ATPase, depending on the vessel studied in a way similar to the changes observed in ouabain-sensitive Na^+,K^+-ATPase activity. Thus, in the thoracic aorta from ouabain-treated rats both α_1 and α_2 expressions were increased; in superior mesenteric arteries, no modification of either isoform was found, while in the tail artery from ouabain-treated rats, the α_1 and α_2 protein expression was reduced. In agreement, a recent report suggests that chronic treatment with either ouabain or digoxin induces tissue specific differential regulation of the Na^+ pump α subunit mRNA (Wang *et al*., 2000). In addition, earlier reports had already suggested alterations of the expression of α isoforms in various pathologies, including hypertension (Herrera *et al*., 1988; Sahin-Erdemli *et al*., 1995). An upregulation of the α_1 and α_2-subunit isoforms of Na^+,K^+-ATPase has been reported after 1 to 4 days of ouabain treatment in a culture of aortic smooth muscle cells (Liu & Songu-Mize, 1998). An upregulation was also obtained in cultured aortic smooth muscle cells by mechanical strain (Songu-Mize *et al*., 1996), which is another procedure that increases intracellular Na^+, and these increases stimulate the transcription of α_1 and α_2 subunits of the sodium pump in smooth muscle cell cultures (Yamamoto *et al*., 1994).

We do not know where the changes in Na^+ pump are localized in the vascular wall. However, given the higher amount of smooth muscle cells when compared with the endothelium, it seems likely that the altered protein expression could occur in the muscular layer. On the other hand, it is difficult to explain the different effects of ouabain treatment in the three vessels studied. Some investigators have suggested that the activity of Na^+ pump in thoracic aorta, mesenteric and tail artery is inversely correlated with the catecholamine content (Myers *et al*., 1987). It is possible that differences in adrenergic innervation as well as structural differences between blood vessels could be related to the different changes observed in Na pump activity and expression in this hypertensive model.

The regional alterations of the ouabain-sensitive Na^+,K^+-ATPase activity and protein expression might help explain the differences in the phenylephrine-induced contraction observed in the vessels from ouabain-induced hypertensive rats. The increase in the ouabain sensitive Na^+,K^+-ATPase activity that would promote hyperpolarization, together with the increase of endothelial modulation, would also result in the reduction of both maximal response and sensitivity to phenylephrine observed in aorta from ouabain-treated rats. In the tail artery, the activity of ouabain-sensitive Na^+,K^+-ATPase was reduced, leading to higher intracellular Na^+ and Ca^{2+} concentrations, thus increasing contractile activity. However, the enhanced endothelial function observed in this vessel counteracted the contractile activity resulting in a similar phenylephrine-induced contraction in control and ouabain-treated rats. In mesenteric arteries, where endothelial modulation is increased but changes in Na^+ pump activity are not observed, only the maximal response was reduced by ouabain treatment.

In conclusion, the results described here indicate that chronic administration of ouabain induces hypertension. This finding supports the hypothesis that the endogenous Na^+ pump inhibitor, characterized as ouabain or an isomer (Mathews *et al*., 1991), could be related to the genesis and/

or maintenance of hypertension. Although there are proposals suggesting the involvement of central mechanisms in this hypertension model (Huang *et al*., 1994; Veerasingham & Leenen, 1999; Huang & Leenen, 1999), vascular mechanisms with regional alterations of vasopressor responses to phenylephrine as well as of the ouabain sensitive Na^+ pump activity and protein expression are also present.

This study has been supported by grants from DGICYT (PM 97-008 and BX 2000 0153) and CNPq Brazil (200380/99-0). We are grateful to Dr E. Songu-Mize for kindly providing the technical information for Na^+,K^+-ATPase Western-blot analysis, Dr M.C. Fernández-Criado for the care of animals, Ms A. Lores for her skilful technical assistance and Ms C.F. Warren for her linguistic assistance. The authors dedicate this work to the memory of Prof Jesús Marín López, head of the laboratory where this work was performed, who died on 9 June 2000.

References

ARRIBAS, S., MARÍN, J., PONTE, A., BALFAGÓN, G. & SALAICES, M. (1994). Norepinephrine-induced relaxations in rat aorta mediated by endothelial beta adrenoceptors. Impairment by ageing and hypertension. *J. Pharmacol. Exp. Ther.,* **270,** 520 – 527.

BLANCO, G. & MERCER, R.W. (1998). Isozymes of the Na-K-ATPase: heterogeneity in structure, diversity in function. *Am. J. Physiol.,* **275,** F633 – F650.

BLAUSTEIN, M.P. (1993). Physiological effects of endogenous ouabain: control of intracellular Ca^{2+} stores and cell responsiveness. *Am. J. Physiol.,* **264,** C1367 – C1387.

BUCKALEW, JR. V.M. & HADDY, F.J. (1989). The role of digitalis-like factor in the pathophysiology of hypertension. In *Endocrine Mechanisms in Hypertension*. ed. Laragh, J.H., Brenner, B.M. & Kaplan, N.M. pp 307 – 334. New York: Raven Press.

CARGNELLI, G., TREVISI, L., DEBETTO, P., LUCIANI, S. & BOVA, S. (2000). Effect of long-term ouabain treatment on contractile responses of rat aortae. *J. Cardiovasc. Pharmacol.,* **35,** 538 – 542.

CARRIER, G.O. & WHITE, R.E. (1985). Enhancement of alpha-1 and alpha-2 adrenergic agonist-induced vasoconstriction by removal of endothelium in rat aorta. *J. Pharmacol. Exp. Ther.,* **232,** 682 – 687.

COX, R.M. (1986). Effects of norepinephrine on ^{86}Rb-uptake by arteries from spontaneously hypertensive and Wistar-Kyoto rats. *J. Hypertens.,* **4,** S52 – S54.

DAVEL, A.P.C., ROSSONI, L.V. & VASSALLO, D.V. (2000). Effects of ouabain on the pressor response to phenylephrine and on the sodium pump activity in diabetic rats. *Eur. J. Pharmacol.,* **406,** 419 – 427.

DOHI, Y., KOJIMA, M. & SATO, K. (1996). Endothelial modulation of contractile responses in arteries from hypertensive rats. *Hypertension,* **28,** 732 – 737.

GOTTLIEB, S.S., ROGOWSKI, A.C., WEINBERG, M., KRICHTEN, C.M., HAMILTON, B.P. & HAMLYN, J.M. (1992). Elevated concentration of endogenous ouabain in patients with congestive heart failure. *Circulation,* **86,** 420 – 425.

HAMLYN, J.M., HAMILTON, B.P. & MANUNTA, P. (1996). Endogenous ouabain, sodium balance and blood pressure: a review and a hypothesis. *J. Hypertens.,* **14,** 151 – 171.

HAMLYN, J.M., RINGEL, R., SCHAEFFER, J., LEVINSON, P.D., HAMILTON, B.P., KOWARSKI, A.A. & BLAUSTEIN, M.P. (1982). A circulating inhibitor of ($Na^+ + K^+$) ATPase associated with essential hypertension. *Nature,* **300,** 650 – 652.

HERRERA, V.L.M., CHOBANIAN, A.V. & RUIZ-OPAZO, N. (1988). Isoform-specific modulation of Na^+,K^+-ATPase α-subunit gene expression in hypertension. *Science,* **241,** 221 – 223.

HUANG, B.S., HUANG, X., HARMSEN, E. & LEENEN, F.H.H. (1994). Chronic central versus peripheral ouabain, blood pressure, and sympathetic activity in rats. *Hypertension,* **23,** 1087 – 1090.

HUANG, B.S. & LEENEN, F.H.H. (1996). Brain 'ouabain' and angiotensin II in salt-sensitive hypertension in spontaneously hypertensive rats. *Hypertension,* **28,** 1005 – 1012.

HUANG, B.S. & LEENEN, F.H.H. (1999). Brain renin-angiotensin system and ouabain-induced sympathetic hyperactivity and hypertension in Wistar rats. *Hypertension,* **34,** 107 – 112.

KIMURA, K., MANUNTA, P., HAMILTON, B.P. & HAMLYN, J.M. (2000). Different effects of in vivo ouabain and digoxin on renal artery function and blood pressure in the rat. *Hypertens. Res.,* **23,** S67 – S76.

LI, M., MARTIN, A., WEN, C., TURNER, S.W., LEWIS, L.K. & WHITORTH, J.A. (1995). Long-term ouabain administration does not alter blood pressure in conscious Sprague-Dawley rats. *Clin. Exp. Pharmacol. Physiol.,* **22,** 919 – 923.

LIU, X. & SONGU-MIZE, E. (1998). Effects of Na^+,K^+-ATPase α-subunit expression and Na^+-pump activity in aortic smooth muscle cells. *Eur. J. Pharmacol.,* **351,** 113 – 119.

LUDENS, J.H., CLARK, M.A., ROBINSON, F.G. & DUCHARME, D.W. (1992). Rat adrenal cortex is a source of a circulating ouabain-like compound. *Hypertension,* **19,** 721 – 724.

MANUNTA, P., HAMILTON, J., ROGOWSKI, A.C., HAMILTON, B.P. & HAMLYN, J.M. (2000). Chronic hypertension induced by ouabain but not digoxin in the rat: antihypertensive effect of digoxin and digitoxin. *Hypertens. Res.,* **23,** S77 – S85.

MANUNTA, P., ROGOWSKI, A.C., HAMILTON, B.P. & HAMLYN, J.M. (1994). Ouabain-induced hypertension in the rat: relationships among plasma and tissue ouabain and blood pressure. *J. Hypertens.,* **12,** 549 – 560.

MARÍN, J. & REDONDO, J. (1999). Vascular sodium pump: endothelial modulation and alterations in some pathological processes and aging. *Pharmacol. Ther.,* **84,** 249 – 271.

MARÍN, J. & RODRÍGUEZ-MARTÍNEZ, M.A. (1997). Role of vascular nitric oxide in physiological and pathological conditions. *Pharmacol. Ther.,* **75,** 111 – 134.

MARÍN, J., SÁNCHEZ-FERRER, C.F. & SALAICES, M. (1988). Effects of ouabain on isolated cerebral and femoral arteries of the cat: a functional and biochemical study. *Br. J. Pharmacol.,* **93,** 43 – 52.

MATHEWS, W.R., DUCHARME, D.W., HAMLYN, J.M., HARRIS, D.W., MANDEL, F., CLARK, M.A. & LUDENS, J.H. (1991). Mass spectral characterization of an endogenous digitalislike factor from human plasma. *Hypertension,* **17,** 930 – 935.

MYERS, J.H., LAMB, F.S. & WEBB, R.C. (1987). Contractile response to ouabain and potassium-free solution in vascular tissue from renal hypertensive rats. *J. Hypertens.,* **5,** 161 – 171.

ORIOWO, M.A., BEVAN, J.A. & BEVAN, R.D. (1987). Variation in sensitivity of *alpha* adrenoceptor-mediated contraction of the vascular smooth muscle of rabbit elastic and muscular arteries is related to receptor affinity. *J. Pharmacol. Exp. Ther.,* **241,** 239 – 244.

OVERBECK, H.W., PAMNANI, M.B., AKERA, T., BRODY, T.H. & HADDY, F.J. (1976). Depressed function of ouabain-sensitive sodium-potassium pump in blood vessels from renal hypertensive dogs. *Circ. Res.,* **38,** 48 – 52.

PACHECO, M.E., MARÍN, J., MANSO, A.M., RODRÍGUEZ-MARTÍNEZ, M.A., BRIONES, A., SALAICES, M. & REDONDO, J. (2000). Nitric oxide synthase induction by ouabain in vascular smooth muscle cells from normotensive and hypertensive rats. *J. Hypertens.,* **18,** 877 – 884.

PIDGEON, G.B., RICHARDS, A.M., NICHOLLS, M.G., CHARLES, C.J., RADEMAKER, M.Y., LYNN, K.L., BAILEY, R.R., LEWIS, L.K. & YANDLE, T.G. (1996). Chronic ouabain infusion does not cause hypertension in sheep. *Am. J. Physiol.,* **270,** E386 – E392.

PONTE, A., MARÍN, J., ARRIBAS, S., GONZÁLEZ, R., BARRÚS, M.T., SALAICES, M., SÁNCHEZ-FERRER, C.F. (1996). Endothelial modulation of ouabain-induced contraction and sodium pump activity in the aorta of normotensive Wystar-Kyoto rats and spontaneously hypertensive rats. *J. Vasc. Res.,* **33,** 164 – 174.

REDONDO, J., PEIRÓ, C., RODRÍGUEZ-MAÑAS, L., SALAICES, M., MARÍN, J. & SÁNCHEZ-FERRER, C.F. (1995). Endothelial stimulation of the sodium pump in cultured vascular smooth muscle. *Hypertension,* **26,** 177 – 185.

RODRÍGUEZ-MAÑAS, L., PAREJA, A., SÁNCHEZ-FERRER, C.F., CASADO, M.A., SALAICES, M. & MARÍN, J. (1992). Endothelial role in ouabain-induced contractions in guinea pig carotid arteries. *Hypertension,* **20,** 674 – 681.

ROSSONI, L.V., CUNHA, V., FRANÇA, A. & VASSALLO, D.V. (1999). The influence of nanomolar ouabain on vascular pressor responses is modulated by the endothelium. *J. Cardiovasc. Pharmacol.,* **34,** 887–892.

SAHIN-ERDEMLI, I., MEDFORD, R.M. & SONGU-MIZE, E. (1995). Regulation of Na^+,K^+-ATPase α-subunit isoforms in rat tissues during hypertension. *Eur. J. Pharmacol.,* **292,** 163–171.

SAHIN-ERDEMLI, I., RASHED, S.M. & SONGU-MIZE, E. (1994). Rat vascular tissues express all three α-isoforms of Na^+,K^+-ATPase. *Am. J. Physiol.,* **266,** H350–H353.

SONGU-MIZE, E., LIU, X., STONES, J.E. & HYMEL, L.J. (1996). Regulation of Na^+,K^+-ATPase α-subunit expression by mechanical strain in aortic smooth muscle cells. *Hypertension,* **27,** 827–832.

SWEADNER, K.J. (1989). Isozymes of the Na^+/K^+-ATPase. *Biochim. Biophys. Acta,* **988,** 185–220.

TABERNERO, A., GIRALDO, J. & VILA, E. (1996). Effect of N^G-nitro-L-arginine methylester (L-NAME) on functional and biochemical α_1-adrenoceptor-mediated responses in rat blood vessels. *Br. J. Pharmacol.,* **117,** 757–763.

VASSALLO, D.V., SONGU-MIZE, E., ROSSONI, L.V. & AMARAL, S.M.C. (1997). Effects of ouabain on vascular reactivity. *Braz. J. Med. Biol. Res.,* **30,** 545–552.

VEERASINGHAM, S.J. & LEENEN, F.H.H. (1999). Ouabain- and central sodium-induced hypertension depend on the ventral anteroventral third ventricle region. *Am. J. Physiol.,* **276,** H63–H70.

WANG, H., YUAN, W.Q. & LU, Z.R. (2000). Differential regulation of the sodium pump alpha-subunit isoform gene by ouabain and digoxin in tissues of rats. *Hypertens. Res.,* **23,** S55–S60.

WEBB, R.C. & BOHR, D.F. (1978). Potassium-induced relaxations as an indicator of Na^+,K^+-ATPase activity in vascular smooth muscle. *Blood Vessels,* **15,** 198–207.

WEBB, R.C. & BOHR, D.F. (1980). A comparative and regional study of potassium-induced relaxation of vascular smooth muscle. *J. Comp. Physiol.,* **135,** 357–363.

XIE, J., WANG, Y., SUMMER, W.R. & GREENBERG, S.S. (1993). Ouabain enhances basal release of nitric oxide from carotid artery. *Am. J. Med. Sci.,* **305,** 157–163.

YAMAMOTO, K., IKEDA, U., OKADA, K., SAITO, T., KAWAKAMI, K. & SHIMADA, K. (1994). Sodium ion mediated regulation of Na/K-ATPase expression in vascular smooth muscle cells. *Cardiovasc. Res.,* **28,** 957–962.

YUAN, C.M., MANUNTA, P., HAMLYN, J.M., CHEN, S., BOHEN, E., YEUN, J., HADDY, F.J. & PAMNANI, M.B. (1993). Long-term ouabain administration produces hypertension in rats. *Hypertension,* **22,** 178–187.

(Received June 6, 2001
Revised November 13, 2001
Accepted November 13, 2001)