Chronic ouabain treatment exacerbates blood pressure elevation in spontaneously hypertensive rats: the role of vascular mechanisms

Fabiano E. Xavier[b], Ana Paula C. Davel[a], Lívia E. Fukuda[a] and Luciana V. Rossoni[a]

Objective **Hypertensive rats are more sensitive to the pressor effects of acute ouabain than normotensive rats. We analyzed the effect of chronic ouabain (~8.0 μg/day, 5 weeks) treatment on the blood pressure of spontaneously hypertensive rats (SHRs) and Wistar–Kyoto rats and the contribution of vascular mechanisms.**

Methods **Responses to acetylcholine and phenylephrine were analyzed in isolated tail arteries. Protein expression of endothelial nitric oxide synthase and cyclooxygenase-2 (COX-2) were also investigated.**

Results **Ouabain treatment enhanced blood pressure only in SHRs. The pD_2 for acetylcholine was decreased in arteries from SHRs compared with Wistar–Kyoto rats, and ouabain did not change this parameter. However, ouabain was able to increase the pD_2 to phenylephrine in SHRs. Nitric oxide synthase inhibition with N^G-nitro-L-arginine methyl ester or potassium channel blockade by tetraetylamonium increased the response to phenylephrine in SHRs, with a smaller increase in response observed in ouabain-treated SHRs. In addition, indomethacin (a COX inhibitor) and ridogrel (a thromboxane A_2 synthase inhibitor and prostaglandin H_2/thromboxane A_2 receptor antagonist) decreased contraction to phenylephrine in tail rings from ouabain-treated SHRs. Protein expression of endothelial nitric oxide synthase was unaltered following ouabain treatment in SHRs, whereas COX-2 expression was increased.**

Conclusion **Chronic ouabain treatment further increases the raised blood pressure of SHRs. This appears to involve a vascular mechanism, related to a reduced vasodilator influence of nitric oxide and endothelium-derived hyperpolarizing factor and increased production of vasoconstrictor prostanoids by COX-2. These data suggest that the increased plasma levels of ouabain could play an important role in the maintenance of hypertension and the impairment of endothelial function.** ***J Hypertens*** **27:1233–1242 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Abbreviations: ANOVA, analysis of variance; COX, cyclooxygenase; dAUC, difference of area under the concentration–response curves; EDHF, endothelium-derived hyperpolarizing factor; EDTA, ethylenediaminetetraacetic acid; E_{max}, maximum response; eNOS, endothelial nitric oxide synthase; KHB, Krebs–Henseleit bicarbonate buffer; L-NAME, N^G-nitro-L-arginine methyl ester; NO, nitric oxide; NOS, nitric oxide synthase; PAGE, polyacrylamide gel; pD_2, negative logarithm of concentrations producing 50% of maximum response; PGH_2, prostaglandin H_2; SDS, sodium lauryl sulfate; SHR, spontaneously hypertensive rats; TEA, tetraethylammonium; TxA_2, thromboxane A_2; WKY, Wistar–Kyoto rats

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Introduction

Endogenous cardiotonic steroids are thought to play an important role in health and disease [1–3]. Ouabain and marinobufagenin are two such steroids identified in plasma from humans and animals, which are thought to act via Na^+, K^+-ATPase [2–4]. In addition, these compounds have been implicated in the pathogenesis of various disorders involving the renal, nervous and cardiovascular systems.

It is well established that endogenous ouabain plays an important role in the development or maintenance or both of arterial hypertension [1–3]. Evidence suggests that circulating levels of ouabain are elevated in several forms of hypertension [1,5–7]. Both the short-term and long-term treatment of rats with low doses of ouabain lead to development of hypertension [8–18], which can be associated with an increase in sympathetic tone [8,19–21].

The development of hypertension can also lead to peripheral vascular changes [10,12,13,15–18]. Acute administration of 10 nmol/l of ouabain increases the vascular responsiveness to phenylephrine in isolated tail arteries [22], whereas doses approximately 1000-fold higher produce vasoconstriction [23]. Hypertension induced by 5 weeks of ouabain treatment is also followed by functional and structural changes in the vascular wall. We

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have previously demonstrated that in Wistar rats made hypertensive by 5 weeks of ouabain treatment, conductance vessels exhibit reduced vasoconstriction to α-adrenergic agonists and increased activity and expression of Na^+, K^+-ATPase [12,13]. These changes are associated with increased endothelium-derived nitric oxide production as a result of higher expression of endothelial (eNOS) and neuronal (nNOS) nitric oxide synthase [13], which appears to be mediated by activation of the vascular endothelin system [17]. In resistance vessels, ouabain-induced hypertension did not cause changes in the vasoconstrictor response to noradrenaline [16], although structural alterations characterized by inward remodeling have been observed [18].

Several studies [11,14,24–29] suggest that hypertensive rats are more sensitive to the acute pressor effect of ouabain both *in vivo* and *in vitro*. Previous results from our group revealed that the acute hypertensive effect of ouabain (administered at a nanomolar range) and the potentiator effect on phenylephrine-induced vasoconstriction was larger in spontaneously, N^G-nitro-L-arginine methyl ester (L-NAME), deoxycorticosterone acetate (DOCA)-salt and one kidney, one clip (1K1C) hypertensive rats compared with their respective normotensive controls [11,14,25,28,29]. These results suggest that vascular beds from hypertensive rats show increased sensitivity to ouabain and led us to hypothesize that long-term treatment with ouabain might also augment the increase in blood pressure (BP) of spontaneously hypertensive rats (SHRs).

Therefore, the aim of our study was to assess the effects of long-term ouabain treatment on the BP of SHR and Wistar–Kyoto (WKY) rats and analyze whether this effect was associated with changes in vascular reactivity via activation of α_1-adrenoceptors.

Methods

Animal housing

Six-week-old male WKY and SHRs were obtained from colonies maintained at the Animal Quarters of the Institute of Biomedical Sciences of the University of Sao Paulo. Rats were housed at a constant room temperature, humidity and light cycle (12 : 12 h light dark), with free access to standard rat chow and tap water. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and with the guidelines of the Committee on Care and Use of Laboratory Animal Resources of the Institute of Biomedical Sciences of the University of Sao Paulo.

Pellet implantation

Rats were divided into four experimental groups: untreated WKY and SHRs (received vehicle) and ouabain-treated WKY and SHRs (~8.0 μg/day, subcutaneously). Animals were anesthetized with ketamine, xylazine and acepromazine mixture [64.9, 3.2 and 0.78 mg/kg, respectively, intraperitoneally (i.p.)] and a small incision was made in the back of the neck to implant a subcutaneous controlled time-release pellet (Innovative Research of America) containing ouabain (0.5 mg) or vehicle (placebo), as previously described [8,12,13,15–18].

After 5 weeks of ouabain or vehicle treatments, the following protocols were performed.

Arterial blood pressure measurement

Rats were anesthetized with ketamine, xylazine and acepromazine mixture (64.9, 3.2 and 0.78 mg/kg, respectively, i.p.) and allowed to breathe room air spontaneously. The right carotid artery was cannulated with a polyethylene catheter (PE-50 with heparinized saline) that was exteriorized in the mid-scapular region. After 24 h, arterial pressure and heart rate were measured in conscious animals by a pressure transducer (model DT-100; Utah Medical Products, Midvale, Utah, USA) and recorded using an interface and software for computer data acquisition (Windaq; Dataq Instruments Inc, Akron, Ohio, USA). Heart rate was determined from the intrabeat intervals.

After obtaining measurements of arterial pressure and heart rate, the rats were once again anesthetized with ketamine, xylazine and acepromazine mixture and killed by exanguination. Tail arteries were then carefully dissected out and cleaned of connective tissue. For reactivity experiments, the arteries were divided into segments of 3 mm in length. For analysis of eNOS and cyclooxygenase-2 (COX-2) protein expression, arteries were rapidly frozen in liquid nitrogen and kept at −70°C until the day of analysis.

Vascular reactivity study

For isometric tension recording, each segment of tail artery was removed and placed in cold oxygenated Krebs–Henseleit bicarbonate buffer (KHB) as previously described by Rossoni *et al.* [12]. The buffer consisted of following elements (in mmol/l): NaCl 118; KCl 4.7; $NaHCO_3$ 25; $CaCl_2.2H_2O$ 2.5; KH_2PO_4 1.2; $MgSO_4.7H_2O$ 1.2; glucose 11 and ethylenediaminetetraacetic acid (EDTA) 0.01. Segments of tail artery (3 mm in length), free of fat and connective tissue, were mounted between two steel hooks in isolated tissue chambers containing 5 ml of gassed (95% O_2 and 5% CO_2) KHB, at 37°C, under a resting tension of 0.5 g. Isometric tension was recorded by using an isometric force displacement transducer (Letica TRI 210, Barcelona, Spain) connected to an acquisition system (MP100; Biopac Systems, Santa Barbara, California, USA).

After a 45 min equilibration period, arteries were twice exposed to KCl (75 mmol/l) to assess maximum contractility. After 30 min, rings were contracted with a concentration of phenylephrine inducing 50–70% of the contraction induced by KCl. Acetylcholine (1 nmol/l–10 μmol/l) was then added to assess endothelium-dependent relaxation. After 60 min, cumulative concentration–response curves for phenylephrine (0.1 nmol/l–300 μmol/l) were generated. In certain 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 to acetylcholine (10 μmol/l) in rings precontracted with phenylephrine. Additionally, the effects of the nonselective NOS inhibitor L-NAME (100 μmol/l), the potassium channel blocker tetraethylammonium (TEA, 5 mmol/l), the COX inhibitor indomethacin (10 μmol/l) or the thromboxane (TxA_2) synthase inhibitor and prostaglandin H_2 (PGH_2)/TxA_2 receptor antagonist, ridogrel (1 μmol/l) on concentration–response curves for phenylephrine were investigated. All drugs were added 30 min before generating the concentration–response curve.

Western blot analysis

For analysis of eNOS and COX-2 expression, tail arteries were dissected out, cleaned of connective tissue, frozen in liquid nitrogen and kept at −70°C until the day of analysis. Proteins from homogenized [Tris, 10 mmol/l (pH 7.4), sodium lauryl sulfate (SDS), 1% and sodium metavanadate, 1 mmol/l] tail arteries (100 μg per lane) were separated by 7.5% sodium lauryl sulfate-polyacrylamide gel (SDS-PAGE) and then transferred to polyvinyl difluoride membranes overnight. Membranes were then incubated for 12 h at 4°C with monoclonal antibodies against eNOS (1 : 1000; BD Transduction Laboratories, San Jose, California, USA) or COX-2 (2.5 μg/ml; Upstate Biotechnology, Lake Placid, New York, USA). After washing, the membranes were incubated for 2 h with antirabbit (1 : 1500, Bio-Rad Laboratory, Hercules, California, USA) or antigoat (1 : 1500, Santa Cruz Laboratory, Santa Cruz, California, USA) IgG antibodies conjugated to horseradish peroxidase to detect eNOS or COX-2, respectively. The immunocomplexes were detected using an enhanced horseradish peroxidase/luminol chemiluminescence system (ECL Plus; Amersham International plc, Little Chalfont, UK) and subjected to autoradiography (Hyperfilm ECL; Amersham International plc). Signals on the immunoblot were quantified using a computer program (Scion Image, Frederick, Maryland, USA). The same membranes were used to determine α-actin protein expression using a mouse monoclonal antibody (1 : 1500, Sigma, St Louis, Missouri, USA). Homogenates from endothelial and polymorphonuclear cells were used for eNOS and COX-2-positive control, respectively.

Drugs

All drugs were obtained from Sigma. Stock solutions (10 mmol/l) were prepared in distilled water, except for indomethacin, which was dissolved in 0.1 mol/l Tris (hydroxymethyl aminomethane) buffer, and ridogrel, which was dissolved in physiological saline containing Na_2CO_3 (2%) and NaOH (40 mmol/l). Stock solutions were kept at −20°C and appropriate dilutions were made on the day of the experiment.

Statistical analysis

Contractile responses are expressed as a percentage of the maximum response produced by KCl. For each concentration–response curve, the maximum effect (E_{max}) and the concentration of agonist producing half of the E_{max} (log EC_{50}) were calculated using nonlinear regression analysis (GraphPad Prism Software, San Diego, California, USA). In order to compare the effects of the different drugs or endothelium denudation on the contractile responses to phenylephrine, results were expressed as 'differences' of area under the concentration–response curves (dAUC) to phenylephrine in control and experimental situations. AUCs were calculated from the individual concentration–response curve plot (GraphPad Prism Software) with the differences expressed as a percentage of the difference in the AUC from the corresponding control situation.

COX-2 and eNOS expressions are expressed as the ratio between optical density for eNOS or COX-2 and the signal for α-actin.

All values are expressed as means ± SEM. Results were analyzed using unpaired Student's *t*-test or by completely randomized two-way analysis of variance (ANOVA). When ANOVA showed a significant effect of treatment, Bonferroni's post-hoc test was used to compare individual means. Differences were considered statistically significant at P value less than 0.05.

Results

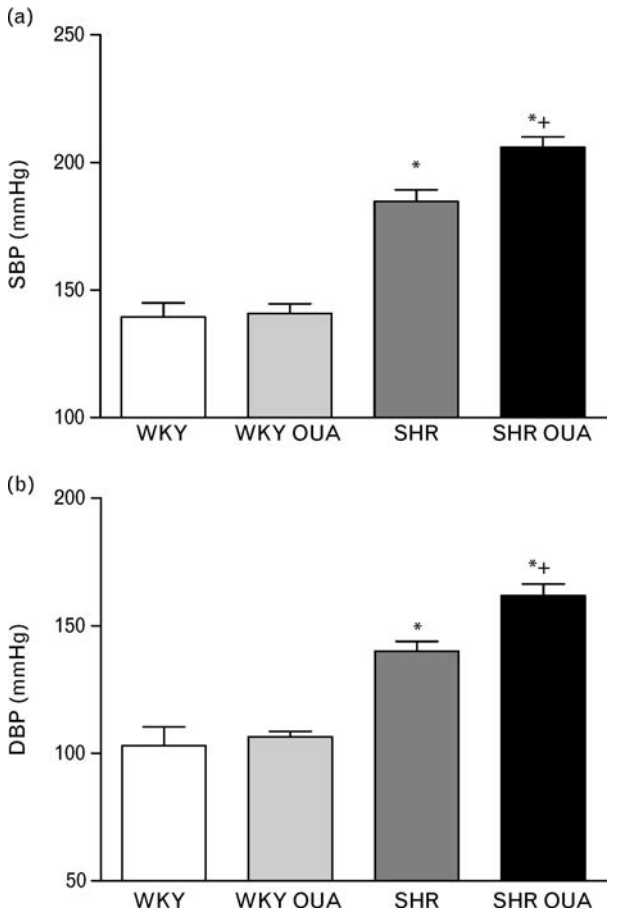
Hemodynamic parameters and body weight

As expected, SHRs exhibited higher systolic and diastolic BP in comparison with WKY rats (Fig. 1a and b). After 5 weeks of ouabain treatment, both the systolic and diastolic BPs were significantly increased in SHRs compared with controls (Fig. 1a and b). However, no changes in systolic or diastolic BP were observed in WKY rats following 5 weeks of ouabain treatment (Fig. 1a and b). In both SHR and WKY rats, heart rate and body weight were not changed by ouabain treatment (results not shown).

Vascular reactivity

Responses to KCl (75 mmol/l) in tail arteries from ouabain-treated WKY and SHRs were similar to those obtained in vessels from the corresponding untreated

Fig. 1



Values of systolic (SBP) and diastolic (DBP) blood pressure in normotensive Wistar–Kyoto (WKY, $N = 6–7$) and in spontaneously hypertensive rats (SHRs, $N = 10–11$) that received vehicle (untreated) or ouabain (OUA) subcutaneously for 5 weeks. Results are expressed as means ± SEM for the number of animals used. Analysis of variance (ANOVA; two-way): $^{*}P < 0.05$ vs. WKY and $^{+}P < 0.05$ vs. untreated.

rats (SHR: 0.72 ± 0.04 vs. SHR/ouabain: 0.78 ± 0.04 g; WKY: 0.76 ± 0.04 vs. WKY/ouabain: 0.70 ± 0.03 g, $P > 0.05$). The sensitivity (pD_2) but not the maximal response (E_{max}) to acetylcholine-induced endothelium-dependent relaxation was decreased in arteries from untreated SHRs compared with WKY rats (pD_2: WKY: 6.80 ± 0.01 vs. SHR: 6.60 ± 0.02, $P < 0.05$; Fig. 2a and c). However, relaxation to acetylcholine remained unaltered in both SHR and WKY rats following ouabain treatment (Fig. 2a and c).

Contractile responses to phenylephrine were similar in arteries from untreated WKY and SHRs (pD_2: WKY: 6.07 ± 0.01 vs. SHR: 5.65 ± 0.08, $P > 0.05$; E_{max}: WKY: 119 ± 0.70 vs. SHR: $118 \pm 2.97\%$ to KCl, $P > 0.05$). In arteries from WKY rats, this response was not altered by ouabain treatment (Fig. 2b), whereas in arteries from SHRs, the pD_2 to phenylephrine was increased with ouabain treatment without changes in E_{max} (Fig. 2d, Table 1). As ouabain treatment had no statistically significant effect on the vascular reactivity of WKY arteries, the following experiments were performed only with vessels from SHRs.

Endothelium denudation did not significantly alter pD_2 or E_{max} to phenylephrine in tail arteries from either control or ouabain-treated SHRs (Fig. 3a and b, dAUC graph between Fig. 3a and b, Table 1).

To assess the contribution of endothelium-derived nitric oxide to the phenylephrine responses, arteries were incubated with the NOS inhibitor L-NAME. In both groups, pretreatment of tail arteries with L-NAME resulted in leftward shifts of the concentration–response curves to phenylephrine (Fig. 3c and d, Table 1). However, the potentiation induced by L-NAME was smaller in ouabain-treated SHRs compared with controls (see dAUC graph between Fig. 3c and d).

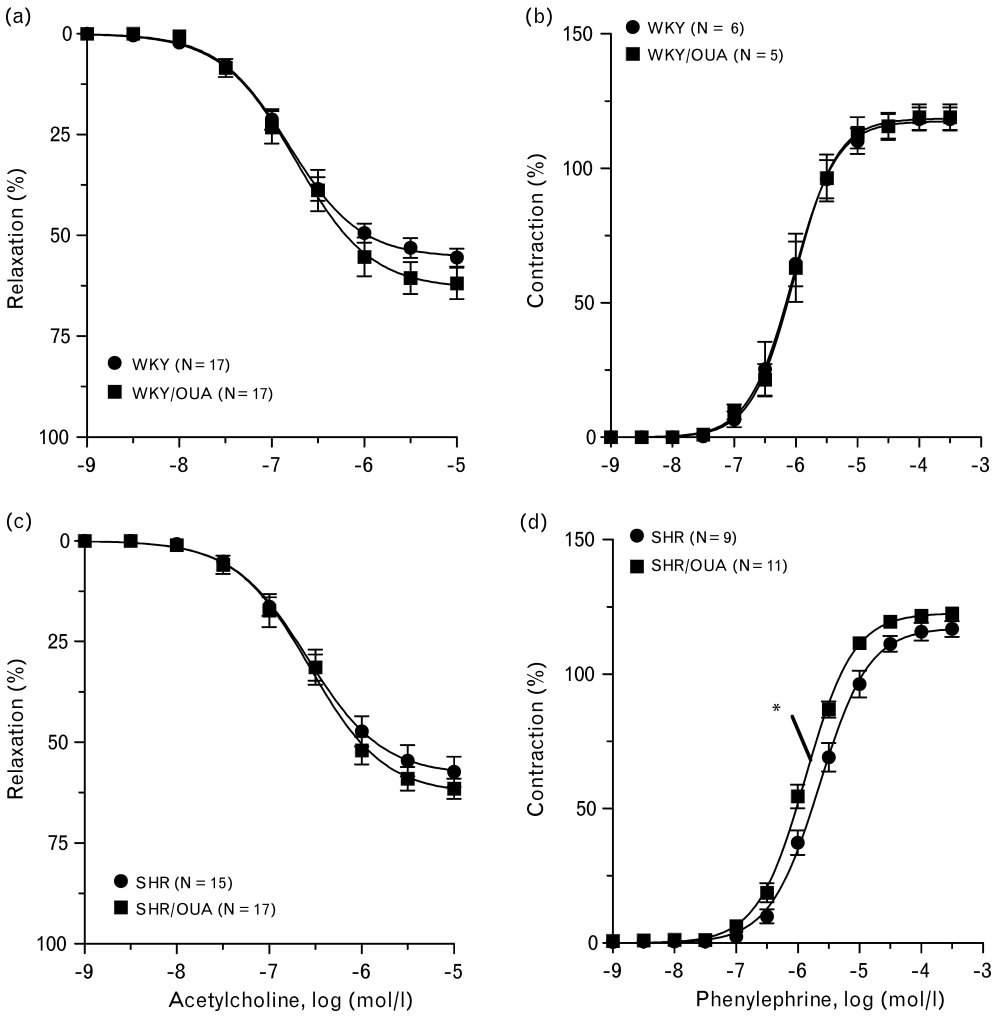
In order to analyze the role of endothelium-dependent hyperpolarizing factor (EDHF) in the effects of ouabain on the phenylephrine response, segments from tail arteries were preincubated with TEA. In vessels from untreated SHRs, pretreatment with TEA resulted in an increase in pD_2 without significant changes in the E_{max} to phenylephrine (Fig. 3e, Table 1). However, in segments from ouabain-treated SHRs, the increase in pD_2 was smaller and accompanied by a reduction in the E_{max} response to phenylephrine (Fig. 3f, see dAUC graph between Fig. 3e and f, Table 1).

The COX inhibitor indomethacin increased the pD_2 to phenylephrine in arteries from untreated SHRs (Fig. 4a, Table 1), whereas segments from ouabain-treated SHRs showed a reduced E_{max} to phenylephrine in the presence of indomethacin (Fig. 4b, Table 1). Preincubation with ridogrel, a TxA_2 synthase inhibitor and PGH_2/TxA_2 receptor antagonist, did not alter phenylephrine-induced contraction in tail arteries from untreated SHRs (Fig. 4c) but constriction was reduced in segments from ouabain-treated SHRs (Fig. 4d, Table 1). It is interesting to observe that both indomethacin and ridrogel had similar effects on phenylephrine-induced contraction in tail rings from ouabain-treated SHRs (compare dAUC graph between Fig. 4a and b and dAUC graph between Fig. 4c and d).

Western blot analysis of endothelial nitric oxide synthase and cyclooxygenase-2 expression

Figure 5 shows eNOS and COX-2 protein expression detected by western blot in tail arteries from ouabain-treated and untreated SHRs. Protein expression of eNOS was not affected by chronic ouabain treatment (Fig. 5a), but expression of COX-2 was increased in tail arteries from ouabain-treated SHRs in comparison with controls (Fig. 5b).

Fig. 2



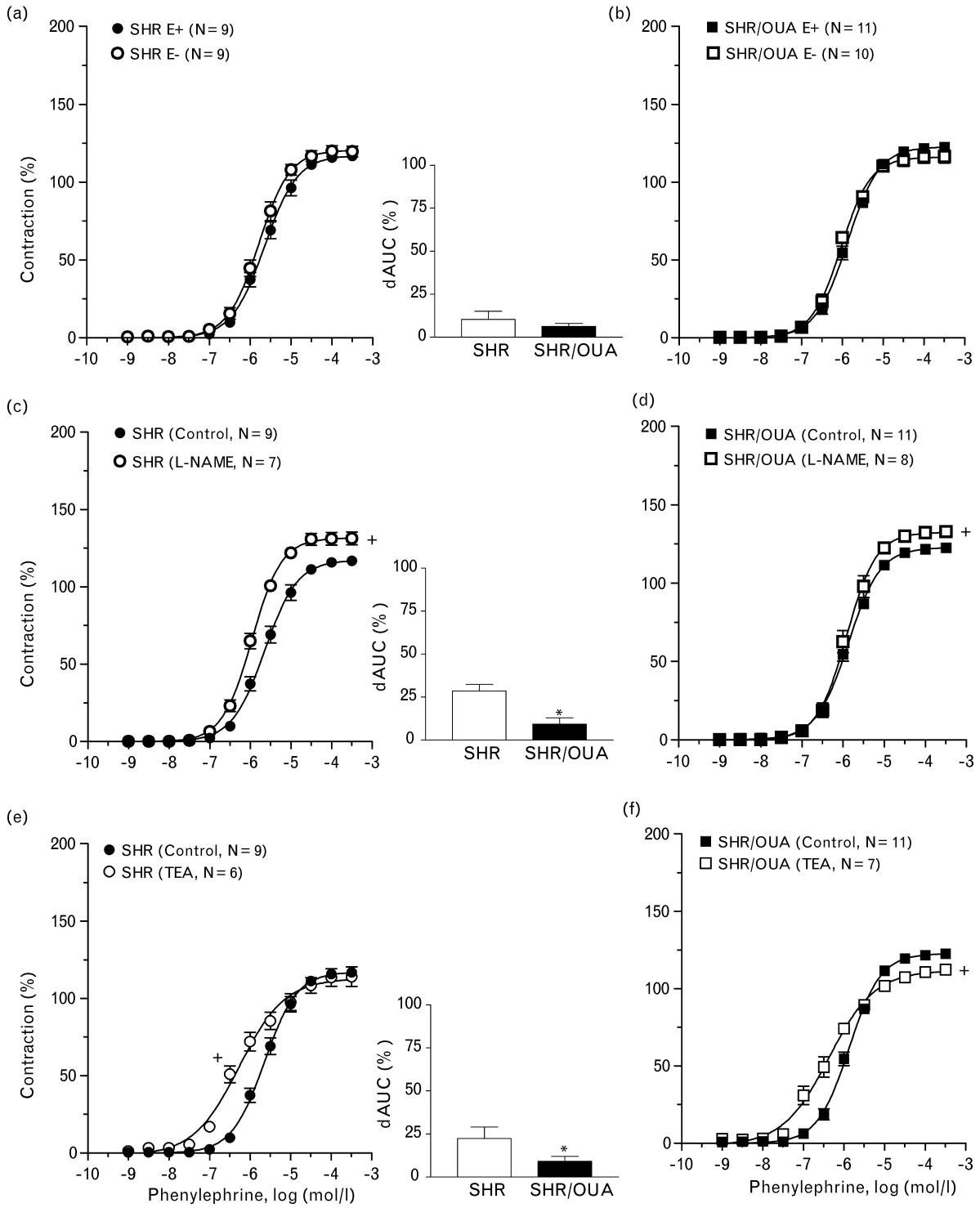
Endothelium-dependent relaxation to acetylcholine and contractile responses to phenylephrine in tail artery from Wistar–Kyoto (WKY; a and b, respectively) and spontaneously hypertensive rats (SHRs; c and d, respectively) that received vehicle (untreated) or ouabain (OUA) subcutaneously for 5 weeks. Results are expressed as means ± SEM for the number of animals used. Analysis of variance (ANOVA; two-way): $^{*}P < 0.05$ vs. untreated animals.

Table 1 Effect of endothelium denudation, N^{G}-nitro-L-arginine methyl ester, tetraethylammonium, indomethacin and ridogrel on the maximal response and pD_2 (−log EC_{50}) to phenylephrine of tail arteries from spontaneously hypertensive rats receiving ouabain (treated) or vehicle (untreated) for 5 weeks

	Untreated		Ouabain-treated	
	E_{max} (%)	pD_2	E_{max} (%)	pD_2
Control (E+)	118 ± 2.97	5.65 ± 0.08 (9)	122 ± 2.53	5.89 ± 0.06* (11)
E−	120 ± 3.60	5.79 ± 0.08 (9)	116 ± 4.54	6.06 ± 0.05 (10)
L-NAME	132 ± 4.30^{+}	5.97 ± 0.06^{+} (7)	132 ± 2.84^{+}	5.93 ± 0.09 (8)
TEA	113 ± 5.37	6.26 ± 0.09^{+} (6)	111 ± 3.50^{+}	6.39 ± 0.08^{+} (7)
Indomethacin	118 ± 1.94	5.94 ± 0.05^{+} (8)	112 ± 2.30^{+}	5.89 ± 0.05 (9)
Ridogrel	114 ± 2.57	5.91 ± 0.10 (5)	109 ± 5.32^{+}	5.79 ± 0.05 (6)

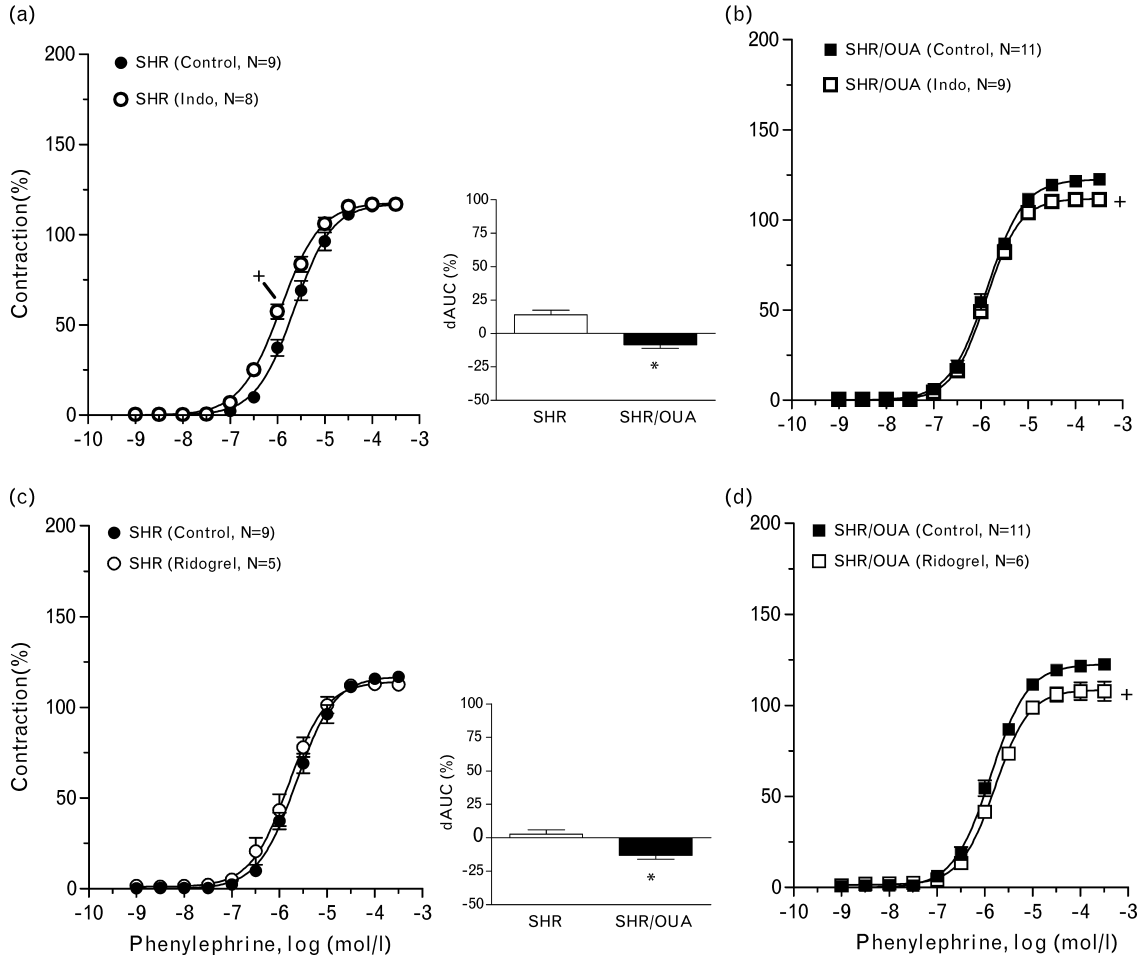
E_{max} expressed as a percentage of contraction to KCl 75 mmol/l. E−, endothelium denuded; E+, endothelium intact; E_{max}, maximum response; L-NAME, N^{G}-nitro-L-arginine methyl ester; pD_2, negative logarithm of concentrations producing 50% of maximum response; TEA, tetraethylammonium. Values are means ± SEM; the number of animals is indicated in parentheses. Analysis of variance (ANOVA). $^{+}P < 0.05$ vs. control, $^{*}P < 0.05$ vs. untreated rats.

Fig. 3



Effect of endothelium denudation (a and b), N^G-nitro-L-arginine methyl ester (L-NAME; c and d) or tetraethylammonium (TEA, e and f) on the concentration-dependent response curves to phenylephrine in tail arteries from ouabain (OUA)-treated and untreated spontaneously hypertensive rats (SHRs). Results (means ± SEM) are expressed as a percentage of response elicited by KCl (75 mmol/l). Analysis of variance (ANOVA; two-way): $^{+}P < 0.05$ vs. SHR or SHR/OUA (control). Insert graph shows differences in area under the concentration–response curve (dAUC) to phenylephrine in endothelium-intact (E+) and endothelium-denuded (E−) arteries or in the absence and in the presence of L-NAME or TEA. dAUC values (means ± SEM) are expressed as a percentage of the difference of the corresponding AUC for segments under control condition (unpaired *t*-test, $^{*}P < 0.05$ vs. SHR).

Fig. 4



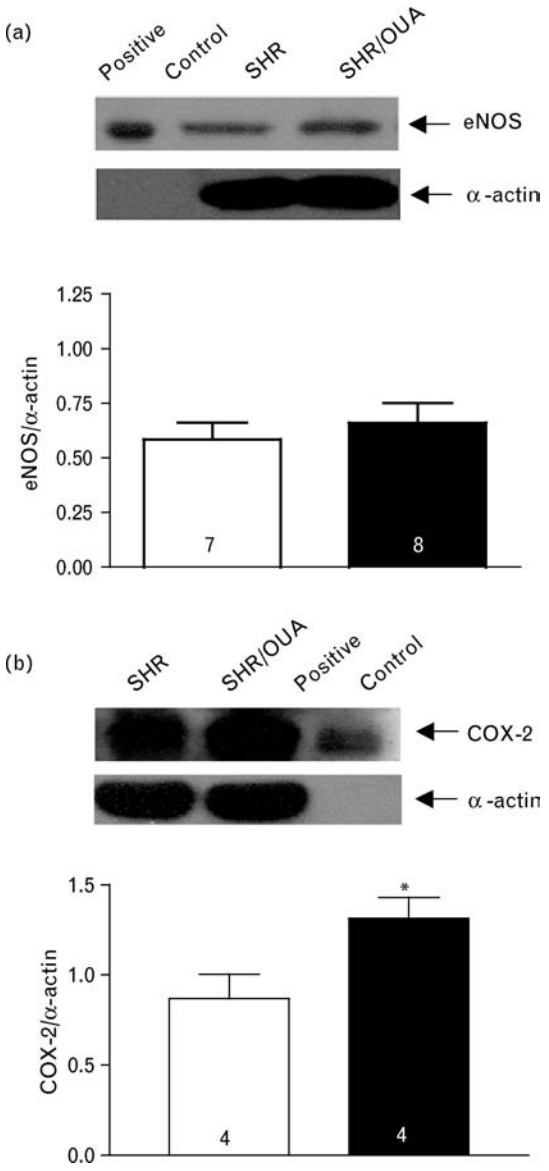
Effect of indomethacin (Indo) or ridogrel on the concentration-dependent curves to phenylephrine in tail arteries from untreated (a and c, respectively) and 5-week ouabain (OUA)-treated (b and d, respectively) spontaneously hypertensive rats (SHRs). Results (means ± SEM) are expressed as a percentage of response elicited by KCl (75 mmol/l). Analysis of variance (ANOVA; two-way): $^{+}P < 0.05$ vs. SHR or SHR/OUA (control). Insert graph shows differences in area under the concentration–response curve (dAUC) to phenylephrine in the absence and in the presence of Indo or ridogrel. dAUC values (means ± SEM) are expressed as a percentage of the difference of the corresponding AUC for segments in absence of drugs (unpaired *t*-test, $^{*}P < 0.05$ vs. SHR).

Discussion

Hypertensive rats are thought to be more sensitive to the pressor effects of acute ouabain than normotensive rats [11,25,28,29]. Evidence suggests that ouabain produces a greater increase in vascular tone in hypertensive rats [11,25,26,28,29] and patients [30] in comparison with their normotensive counterparts. In the present study, we determined whether administration of ouabain over a 5-week period further elevates arterial BP in SHRs and whether this effect is associated with vascular mechanisms.

It is well established that endogenous ouabain behaves as an important modulator of BP [1–3] and chronic ouabain administration in rats leads to development of hypertension [8–10,12,13,15–18]. However, the role of endogenous ouabain in the mechanisms of elevated BP found in hypertensive subjects remains unclear, as changes in the tissue or plasma levels or both of ouabain occur after BP elevation or vascular dysfunction or both. Results from this study demonstrate for the first time that 5 weeks of ouabain treatment causes an additional increase in the BP of SHRs but not in WKY rats, which is in line with previous acute ouabain studies performed in the same strains [28,29]. Furthermore, our results support the hypothesis that increased ouabain levels could contribute to the mechanisms that maintain elevated BP in hypertensive animals.

Fig. 5



Effect of ouabain treatment on endothelial nitric oxide synthase (eNOS) and cyclooxygenase-2 (COX-2) protein expression in tail arteries of spontaneously hypertensive rats (SHRs): (a) upper panel: representative blot for eNOS protein expression in tail arteries from ouabain-treated (OUA) and untreated SHRs. Left lane; corresponding positive control for eNOS protein (endothelial cells). Lower panel: densitometric analysis of the western blot for eNOS protein expression. (b) Upper panel: representative blot for COX-2 protein expression in tail arteries from OUA-treated and untreated SHRs. Right lane; corresponding positive control for COX-2 protein (polymorphonuclear cells). Lower panel: densitometric analysis of the western blot for COX-2 protein expression. Results (means ± SEM) are expressed as the ratio between the signal for the eNOS or COX-2 and the signal for α-actin. Insert numbers in the bar chart indicate the number of animals used. $^{*}P < 0.05$ vs. SHR.

The hypertensive effect of ouabain involves both central and peripheral mechanisms, and is associated with an increase in sympathetic tone [8,19–21], and alterations in vascular structure and function [10,12,13,16–18]. In the current study, we determined whether the effects of ouabain on BP in SHRs were associated with changes in vascular function.

Endothelial dysfunction with impaired nitric oxide production and increased vascular contractility are important pathophysiological elements underlying hypertension [31–34]. We tested the hypothesis that endothelial nitric oxide-dependent vasodilatation and smooth muscle contractility are altered in ouabain-treated SHRs. In line with previous data [12,13,16,17], the present results demonstrate that in tail arteries from WKY and SHRs, neither KCl-induced contraction nor endothelium-dependent vasodilatation to acetylcholine was altered by ouabain treatment. However, vessels from ouabain-treated SHRs showed greater sensitivity to phenylephrine. Accordingly, acute nanomolar concentrations of ouabain are able to enhance phenylephrine-induced contraction only in isolated tail vascular bed from SHRs and not in the normotensive WKY controls [28,29]. Although other mechanisms could contribute to the effects of ouabain in SHRs, such as a neural mechanism [35,36], the results of the present study suggest that the additional BP elevation with ouabain treatment in these rats involves mechanisms associated with changes in vascular function. The study also indicates contrasting effects of chronic ouabain treatment on vascular function in SHRs compared with Wistar rats. In this latter strain, our previous data suggest that changes in vascular function following chronic ouabain treatment serve as a counter-regulatory mechanism to the elevated BP [12,13,16,17].

Endothelial cells play an important role in the regulation of vasoconstrictor responses elicited by different agonists and represent an important target for vascular actions of ouabain. The mechanism of ouabain-induced endothelial modulation is thought to involve the release of nitric oxide or EDHF [12,13,16,17,22]. However, in vessels from hypertensive rats, the acute administration of ouabain induces the release of an endothelium-derived vasoconstrictor factor that acts synergistically with ouabain to increase vascular tone [26,29]. In the present study, endothelial removal, the inhibition of nitric oxide synthesis and blockade of the K_{ca}^{2+} channel were used to assess the contribution of the endothelium, nitric oxide or EDHF to the effects of ouabain treatment in arteries from SHRs.

Removal of the endothelium did not change the vasoconstrictor response to phenylephrine in tail arteries, neither from control nor ouabain-treated SHRs. Similar results were found by Xavier *et al.* [16] using resistance mesenteric arteries from 5-week ouabain-treated Wistar rats. In the presence of the NOS inhibitor L-NAME, the concentration–response curves to phenylephrine were shifted to the left in tail arteries from both

ouabain-treated and control SHRs. However, the increase in phenylephrine response after L-NAME treatment was smaller in vessels from the ouabain-treated SHRs compared with controls. This suggests that ouabain impairs the production or bioavailability or both of nitric oxide in tail arteries from SHRs, which would account for the increased response to phenylephrine observed after ouabain treatment. Vessels from SHRs exhibit impairment in the vascular nitric oxide pathway [34]. In addition, in the current study, western blot analysis revealed no change in eNOS expression in tail arteries from ouabain-treated SHRs, indicating the reduced nitric oxide modulation on the contractile response to phenylephrine could be due to the reduced bioavailability of nitric oxide. Our results indicate increased levels of ouabain in plasma from hypertensive rats further impair the influence of nitric oxide and exacerbate the vascular damage associated with hypertension.

EDHF also plays a role in the vascular effects of ouabain. Rossoni *et al.* [13,22] reported that both acute and chronic ouabain treatment releases an EDHF that appears to open K_{ca}^{2+} channels in tail arteries from Wistar rats. In contrast with these reports, the present study demonstrates that following ouabain treatment, the modulatory effects of EDHF on phenylephrine-induced contraction are impaired in tail arteries from SHRs. This is in line with a previous study on mesenteric resistance arteries from ouabain-treated Wistar rats [16] and suggests an additional mechanism that could contribute to the hyperreactivity to phenylephrine observed in arteries from ouabain-treated SHRs.

Evidence suggests that the vasoconstrictor response to α-adrenergic agonists is largely mediated by COX-derived vasoconstrictor prostanoids [37,38]. Hypertension is thought to modify the role of these products in the vasodilator and vasoconstrictor responses [38–41]. Further to this, it has been reported that ouabain may induce the release of prostanoids from the vascular endothelium [42]. To test the possible involvement of these products in the effects of ouabain on the phenylephrine response in SHRs, we investigated whether indomethacin (a COX inhibitor) or ridogrel (TxA_2 synthase inhibitor and PGH_2/TxA_2 receptor antagonist) affected phenylephrine-induced contraction. In arteries from untreated SHRs, the contraction to phenylephrine was increased by indomethacin, while it remained unchanged in the presence of ridogrel. However, in arteries from ouabain-treated SHRs, both indomethacin and ridogrel decreased the phenylephrine contraction by a similar magnitude. Taken together, our results indicate that in untreated SHRs, the COX pathway modulates the phenylephrine response via a vasodilatory prostanoid, whereas in ouabain-treated SHRs, the response to phenylephrine is modulated mainly by a vasoconstrictor prostanoid, probably TxA_2.

As COX-2 has been found to be overexpressed in vessels from SHRs [38,40], we tested the possibility that changes in the COX pathway following ouabain treatment are related to changes in COX expression. The results obtained show that COX-2 expression was increased in arteries from ouabain-treated SHRs, suggesting that the increased TxA_2 release is probably derived from COX-2 metabolism. Therefore, our results suggest that, in addition to changes observed in nitric oxide and EDHF, an increase in TxA_2 release could play a role in the hyperreactivity to phenylephrine produced by ouabain in tail arteries from SHRs. To our knowledge, this is the first study to demonstrate that in tail arteries from SHRs, chronic ouabain treatment enhances COX-2 expression and increases the participation of TxA_2 in the contractile responses to phenylephrine. These results led us to hypothesize that anti-inflammatory drugs, especially COX-2 inhibitors or TxA_2 receptor antagonists, could ameliorate vascular damage in diseases with elevated levels of ouabain.

In conclusion, our findings demonstrate that ouabain administration further exacerbates the raised BP in SHRs. This suggests that increased ouabain levels could play an important role in the maintenance of hypertension. Our results also demonstrate a role for a vascular mechanism in the effects of ouabain on the elevation of arterial BP in SHRs. This vascular mechanism appears to be related to reduced bioavailability of nitric oxide, decreased EDHF modulation and an increased production of COX2-derived vasoconstrictor factors.

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