Long-Term Ouabain Treatment Impairs Vascular Function in Resistance Arteries

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Key Words

Ouabain-induced hypertension • Resistance arteries • Oxidative stress • COX-2 • Pro-inflammatory cytokines

Abstract

Background/Aims: The purpose of this study was to examine the cardiovascular effects of long-term ouabain treatment at different time points. **Methods:** Systolic blood pressure (SBP) was measured by tail-cuff method in male Wistar rats treated with ouabain (approx. 8.0 $\mu\text{g}\cdot\text{day}^{-1}$) or vehicle for 5, 10 and 20 weeks. Afterwards, vascular function was assessed in mesenteric resistance arteries (MRA) using a wire myograph. ROS production and COX-1 and COX-2, TNF- α , and IL-6 protein expression were investigated. **Results:** SBP was increased by ouabain treatment up to the 6th week and remained stable until the 20th week. However, noradrenaline-induced contraction increased only in MRA in rats treated with ouabain for 20 weeks. NOS inhibition and endothelium removal increased the noradrenaline response, but to a smaller magnitude in MRA in the ouabain group. Moreover, inhibition of COX-2 or incubation with superoxide dismutase restores noradrenaline-induced contraction in the 20-week ouabain group to control levels. ROS production as

well as COX-2, IL-6 and TNF- α protein expression increased in MRA in this group. **Conclusion:** Although ouabain treatment induced hypertension in all groups, a larger noradrenaline induced contraction was observed over 20 weeks of treatment. This vascular dysfunction was related to COX-2-derived prostanoids and oxidative stress, increased pro-inflammatory cytokines and reduced NO bioavailability.

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Introduction

Ouabain is an adrenal- and hypothalamus-derived circulating steroid hormone in humans and animals, which is produced in response to volume expansion, angiotensin II and/or adrenocorticotrophic hormone stimulation [1–4]. It participates in fluid homeostasis and plays a role in the long-term regulation of Na⁺ and K⁺. In addition, ouabain has also been implicated in the control of many functions involving the cardiovascular and nervous systems [2–4], as well as in the pathogenesis of chronic renal failure, hyperaldosteronism, congestive heart failure and hypertension [2, 5, 6].

Treatment with ouabain (or its structurally related analogs) for 2–5 weeks leads to the development of hypertension in rats [7–14]. This is associated with increased sympathetic tone [15–17] and peripheral adjustments in the vascular system [8–14, 18, 19]. Hypertension induced by 5 weeks of ouabain treatment is accompanied by an increase in vasodilator endothelium-derived factors, which act to reduce the vasoconstrictor response to α_1 -adrenergic stimulation and increase the activity of the Na^+K^+ -ATPase in conductance vessels [9–12]. These responses are mediated by an increase in nitric oxide (NO) production, which is thought to be related to overexpression of the eNOS and nNOS isoforms of NO synthase [10, 11]. However, in mesenteric resistance arteries (MRA), ouabain-induced hypertension did not alter the vasoconstrictor response to noradrenaline [12].

Although it is clear that ouabain induces hypertension, it appears that functional changes in the vasculature attempt to counteract the development of hypertension. Exceptions have been observed in the renal artery and vasa recta, as Kimura et al. [8] described an increase in contractile responses and Cao et al. [20] demonstrated endothelial dysfunction. Nevertheless, all these mechanisms were elucidated in animals exposed to a treatment period of 2–5 weeks. In line with these studies, ouabain treatment produces a progressive increase in blood pressure until the 5th week of treatment, but no studies have addressed what happens to blood pressure and vascular function beyond this treatment period.

In view of that, we determined the effect of long-term ouabain treatment on the blood pressure of Wistar rats at different time points, 10 and 20 weeks, and compared the effects with those observed in 5-week ouabain-treated rats. Furthermore, we analyzed if potential time-dependent alterations could occur on the vascular function of resistance arteries, since these arteries are the main control sites of peripheral vascular resistance and consequently of blood pressure. Special attention was paid to the contribution of endothelial factors, oxidative stress and pro-inflammatory cytokines in the altered vascular response.

Methods

Animal Housing

Six-week-old male Wistar rats were obtained from colonies maintained at the Animal House of the Institute of Biomedical Sciences of University of Sao Paulo. Rats were housed in constant room temperature, humidity and light cycles (12:12 h light-dark), with free access to standard rat chow and tap water. The investi-

gation conformed 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.

Rats were divided into 6 experimental groups: vehicle (VHE) and ouabain-treated rats (OUA, approx. $8.0 \mu\text{g}\cdot\text{day}^{-1}$, subcutaneously) during 5, 10 or 20 weeks. Animals were anesthetized with a mixture of ketamine, xylazine and acetopromazin (64.9 , 3.2 and $0.78 \text{ mg}\cdot\text{kg}^{-1}$, respectively, intraperitoneally). A small incision was then made in the back of the neck to implant a subcutaneous controlled time-released pellet (Innovative Research of America, Sarasota, Fla., USA) containing ouabain (0.5 mg) or VHE, as previously described [9–13, 15, 21]. Systolic blood pressure (SBP) was measured by the tail-cuff method (LE 5001; Panlab s.l. Barcelona, Spain) before the start of treatment and every week during treatment.

Vascular Reactivity Measurements

After 5, 10 or 20 weeks of treatment, rats were anesthetized with a mixture of ketamine, xylazine and acetopromazin, and sacrificed by exsanguination. The mesenteric arcade was then carefully removed. Third-order mesenteric arteries [internal diameter; 5 weeks: VHE: 258 ± 5.63 ($n = 15$) versus OUA: $267 \pm 3.97 \mu\text{m}$ ($n = 15$); 10 weeks: VHE: 255 ± 5.01 ($n = 11$) versus OUA: $263 \pm 4.52 \mu\text{m}$ ($n = 17$); 20 weeks: VHE: 274 ± 4.94 ($n = 24$) versus OUA: $273 \pm 5.01 \mu\text{m}$ ($n = 27$), t test, $p > 0.05$] were removed and cleaned of surrounding tissue in cold Krebs-Henseleit solution (KHS, in mmol/l): NaCl 118; KCl 4.7; NaHCO_3 25; $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ 2.5; KH_2PO_4 1.2; $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ 1.2; EDTA 0.01; glucose 11. Segments (2 mm in length) were mounted in a small vessel myograph chamber (Danish Myo Tech, model 610M; JP-Trading I/S) for isometric tension recordings, as described previously [12, 22]. Briefly, two steel wires ($40 \mu\text{m}$ diameter) were introduced through the lumen of the segments and mounted according to the method described by Mulvany and Halpern [22]. After a 15-min equilibration period in oxygenated KHS at 37°C and pH 7.4, segments were stretched to their optimal lumen diameter for active tension development. This was determined based on the internal circumference/wall tension ratio of the segments by setting the internal circumference, L_0 , to 90% of what the vessels would have if they were exposed to a passive tension equivalent to that produced by a transmural pressure of 100 mm Hg (L_{100}) [22]. The diameter (I_1) was determined according to the equation $I_1 = L_1/\pi$, using specific software for normalization of resistance arteries (DMT Normalization Module; ADInstruments). Segments were washed with KHS and left to equilibrate for 30 min. Vessel contractility was then tested by an initial exposure to a high- K^+ (120 mmol/l) solution.

Experimental Protocols

Endothelium-dependent relaxations were studied by evaluating relaxations to acetylcholine (0.01 nmol/l to $30 \mu\text{mol/l}$) in arteries precontracted with a concentration of 9,11-dideoxy-11 α ,9 α -epoxymethanoprostaglandin $\text{F}_{2\alpha}$, (U-46619, a thromboxane A_2 mimetic) to induce 75–100% of the contraction induced by KCl (120 mmol/l). After 30 min, concentration-response curves to noradrenaline (10 nmol/l to $30 \mu\text{mol/l}$) were performed in the absence (E–) and presence (E+) of the endothelium. The endothe-

lium was removed by rubbing the lumen using a human hair previously washed with ethanol (70%) and then with KHS. The endothelium was considered intact if the arteries relaxed more than 80% to acetylcholine, while endothelial denudation was confirmed by less than 10% relaxation. In another set of experiments using E+ rings, we evaluated the effects of the nonselective inhibitor of NOS isozymes, N ω -nitro-L-arginine methyl ester (L-NAME, 100 μ mol/l), the nonselective inhibitor of COX, indomethacin (Indo, 10 μ mol/l), the selective COX-2 inhibitor, N-(2-cyclohexyloxy-4-nitrophenyl) methansulfonamide (NS-398, 10 μ mol/l), the thromboxane A₂ synthesis inhibitor and prostaglandin H₂/thromboxane A₂ receptor antagonist, ridogrel (1 μ mol/l), the superoxide dismutase (SOD, 150 U/ml), or the hydrogen peroxide scavenger, catalase (1,000 U/ml). All drugs were added 30 min before the concentration response curve to noradrenaline and were then maintained throughout the entire experiment.

ROS Measurement in MRA and the Role of COX-2 in the Synthesis of ROS

The oxidative fluorescent dye hydroethidine (Invitrogen, Carlsbad, Calif., USA) was used to evaluate the in situ production of ROS as previously described [23]. Transverse sections (7 μ m) of frozen MRA were collected on glass slides and equilibrated for 10 min in phosphate buffer in a light-protected humidified chamber at 37°C. Fresh buffer containing hydroethidine (5 μ mol/l) in the absence or presence of specific inhibitor of COX-2 (NS-398, 10 μ mol/l) was topically applied to each tissue section and a coverslip placed on top. Slides were incubated in a light-protected humidified chamber at 37°C for 30 min. Negative control sections received the same volume of phosphate buffer but in the absence of hydroethidine. After this period, slides were viewed on an optical microscope (Axioskop; Zeiss) equipped with a filter to rodamin and a camera (ZVS-3C75DE; Zeiss), using an objective for fluorescence at 20 $\times$. Three different areas of each section were analyzed for the presence of ROS by examination of the regions marked with red fluorescence from the oxidation products of dihydroethidium (DHE).

Western Blot Analysis

Proteins (75 μ g) were extracted from third-order branches of the mesenteric artery using a RIPA Lysis Buffer (Upstate). Samples were electrophoretically separated using 7.5 or 12% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Amersham) overnight at 4°C using a Mini Trans-Blot Cell system (Bio-Rad) containing 25 mmol/l Tris, 190 mmol/l glycine, 20% methanol and 0.05% SDS as described previously [10–12, 21].

Membranes were blocked with 5% nonfat dry milk to prevent nonspecific antibody binding and then incubated overnight at 4°C with a primary antibody raised against anti-eNOS (1:1,000 dilution; Transduction Laboratories), anti-COX-1 (1:1,000 dilution; Chemicon), anti-COX-2 (2.5 μ g/ml dilution; Upstate), anti-IL-1 β (1:1,500 dilution; BioLegend), anti-IL-6 (1:1,000 dilution; Abcam) and anti-TNF- α (1:1,500 dilution; BioLegend). Membranes were washed (10 mmol/l Tris, 100 mmol/l NaCl and 0.1% Tween 20), and then incubated with peroxidase-conjugated anti-mouse IgG antibody (1:1,500 dilution; Bio-Rad) for eNOS, peroxidase-conjugated anti-goat IgG antibody (1:15,000 dilution; Jackson ImmunoResearch) for COX-2, peroxidase-conjugated anti-Armenian hamster IgG (1:3,000; Jackson ImmunoResearch) for IL-1 β and TNF- α , or with peroxidase conjugated anti-rabbit IgG

(1:2,000; KPL) for IL-6 and COX-1. Membranes were thoroughly washed, before detecting the immunocomplexes with an enhanced horseradish peroxidase-luminol chemiluminescence system (ECLPlus; Amersham) and subjecting the membrane to autoradiography (Hyperfilm ECL; Amersham). The same membrane was then stripped and used to determine α -actin protein expression using the monoclonal antibody anti- α -actin (1:30,000 dilution; Sigma). Expression of α -actin was then used to normalize the expression of eNOS, COX-1, COX-2, IL-1 β , IL-6 and TNF- α in each sample. Human umbilical vein endothelial cells were used as positive controls for eNOS and lungs of rats previously activated with lipopolysaccharide were used as positive controls for COX-1, COX-2, IL-1 β , IL-6 and TNF- α .

Drugs

Stock solutions of noradrenaline hydrochloride, acetylcholine chloride, N ω -nitro-L-arginine methyl ester, indomethacin, ridogrel, SOD, catalase, U-46619 (Sigma) and NS-398 (Calbiochem-Novabiochem GmbH) were prepared in distilled water. Indomethacin was dissolved in 0.1 mol/l TRIS (hydroxymethyl aminomethane) buffer, while ridogrel was dissolved in saline containing Na₂CO₃ (2%) plus NaOH (40 mmol/l) and U-46619 and NS-398 were dissolved in dimethyl sulfoxide.

Statistical Analysis

All values are expressed as the mean response $\pm$ SEM. The maximal contractile response to noradrenaline (E_{max}) was expressed as percentage of the maximal contraction induced by KCl (120 mmol/l). The negative log of the agonist concentrations resulting in 50% of the maximum response (pD₂) was calculated from concentration-response curves by nonlinear regression analysis of the curve using the computer-based fitting program Graphpad Prism (San Diego, Calif., USA). Protein expression of eNOS, COX-1, COX-2, IL-1 β , IL-6 and TNF- α was expressed as a ratio of the optical density for each protein against α -actin.

Results were analyzed using the unpaired Student's t test or by two-way ANOVA, as appropriate. 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 $p < 0.05$.

Results

Systolic Blood Pressure

Ouabain induced a progressive increase in SBP from the 1st week of treatment, in comparison to VHE-treated animals (fig. 1). At the 6th week of ouabain treatment, SBP values reached a plateau, which then remained stable until the 20th week (fig. 1). The weight gain was similar in each group after 5, 10 or 20 weeks of treatment [5 weeks: VHE: 123 $\pm$ 4 (n = 10) versus OUA: 113 $\pm$ 4 g (n = 10); 10 weeks: VHE: 162 $\pm$ 8 (n = 10) versus OUA: 165 $\pm$ 7 g (n = 10); 20 weeks: VHE: 205 $\pm$ 9 (n = 10) versus OUA: 219 $\pm$ 8 g (n = 10); t test, $p > 0.05$].

Vascular Reactivity Study

KCl (120 mmol/l) evoked similar contractions in MRA from VHE and OUA rats in the three periods of treatment [5 weeks: VHE: 2.53 ± 0.11 (n = 15) versus OUA: 2.32 ± 0.09 mN/mm (n = 15); 10 weeks: VHE: 2.60 ± 0.12 (n = 11) versus OUA: 2.69 ± 0.14 mN/mm (n = 17); 20 weeks: VHE: 2.77 ± 0.16 (n = 24) versus OUA: 2.84 ± 0.14 mN/mm (n = 27); t test, $p > 0.05$]. Similarly, relaxation to acetylcholine was not different between MRA from VHE and OUA rats in any of the groups studied (fig. 2a–c). However, chronic treatment with ouabain promoted an increase in the contractile response to noradrenaline in a time dependent manner (fig. 2d–f). While both 5 and 10 weeks of ouabain treatment did not alter the noradrenaline-induced contraction in MRA [fig. 2d, e; pD_2 : 5 weeks: VHE: 5.97 ± 0.06 (n = 15) versus OUA: 5.94 ± 0.07 (n = 15); 10 weeks: VHE: 6.01 ± 0.08 (n = 11) versus OUA: 5.92 ± 0.08 (n = 17); t test, $p > 0.05$; E_{max} : 5 weeks: VHE: 178 ± 6.65 (n = 15) versus OUA: 173 ± 5.45 % KCl (n = 15); 10 weeks: VHE: 166 ± 5.15 (n = 11) versus OUA: 163 ± 5.20 % KCl (n = 17); t test, $p > 0.05$], 20 weeks of treatment significantly increased both E_{max} and pD_2 (fig. 2f; table 1).

Since the ouabain treatment for minor periods (5 and 10 weeks) had no effect on vascular function in MRA, the experimental protocols described below were performed only in vessels taken from VHE and OUA rats for 20 weeks.

In order to analyze the role of the endothelium and endothelium-derived nitric oxide in noradrenaline responses, experiments were performed in the absence of endothelium or in the presence of the NOS inhibitor (L-NAME, 100 μ mol/l). In both groups, noradrenaline-induced contraction was potentiated by endothelium removal or NOS inhibition; however, this potentiation was smaller in MRA from OUA rats (fig. 3a, b; table 1).

To assess the contribution of superoxide anions to noradrenaline-induced contraction, arteries were incubated with SOD (150 U/ml). In vessels from OUA rats, pretreatment with SOD resulted in a decrease in E_{max} without significant changes in the pD_2 to noradrenaline (fig. 3c; table 1), an effect that was not further modified by co-incubation with SOD and catalase (table 1). In vessels from VHE rats, the response to noradrenaline remained unaltered in the presence of SOD or SOD plus catalase (fig. 3c; table 1).

The COX-1/2 inhibitor indomethacin (10 μ mol/l) and the specific COX-2 inhibitor NS-398 (10 μ mol/l) had no influence on noradrenaline-induced constriction of arteries from VHE rats (fig. 4a; table 1), but re-

Fig. 1. Values of SBP in VHE or OUA-treated rats during 20 weeks. Number of rats used is indicated in parentheses. Results are expressed as means $\pm$ SEM for the number of animals used.

duced constriction of arteries from OUA rats (fig. 4a; table 1). Pre-incubation of vessels with ridogrel, a TxA_2 synthase inhibitor and PGH_2/TxA_2 receptor antagonist, reduced both the E_{max} and the pD_2 of the noradrenaline response in segments from OUA rats, but had no influence on the response of MRA from VHE rats (fig. 4b; table 1).

Western Blot Analysis

Protein expression of eNOS [eNOS/ α -actin: VHE: 0.65 ± 0.09 versus OUA: 0.65 ± 0.05 (n = 6); t test, $p > 0.05$], COX-1 and IL-1 β was similar in MRA from VHE and OUA-treated rats for 20 weeks (fig. 5a, c). However, in MRA from OUA group, protein expression of COX-2, IL-6 and TNF- α was greater when compared to the VHE group (fig. 5b, d, e).

ROS Measurement in MRA

An increase in ethidium bromide fluorescence was observed in MRA from OUA-treated rats for 20 weeks in comparison to VHE rats (fig. 6). Additionally, incubation with COX-2 inhibitor (NS-398, 10 μ mol/l) was able to decrease the ethidium bromide fluorescence in MRA from OUA-treated rats to levels similar to those observed in the VHE group (fig. 6). Moreover, incubation with NS-398 did not change the ethidium bromide fluorescence in arteries from the VHE group (fig. 6).

Fig. 2. Endothelium-dependent relaxation to acetylcholine (a–c) and contractile responses to noradrenaline (d–f) in MRA from VHE and OUA-treated rats for 5 (a, d), 10 (b, e) and 20 (c, f) weeks. Number of rats is indicated in parentheses. Results are expressed as means $\pm$ SEM for the number of animals used.

Table 1. Maximum response and sensitivity

	pD ₂		E _{max} , %	
	VHE	OUA	VHE	OUA
E+	5.92 $\pm$ 0.04 (24)	6.14 $\pm$ 0.05 (27)*	169 $\pm$ 4.57 (24)	190 $\pm$ 6.25 (27)*
E–	6.66 $\pm$ 0.08 (7) ⁺	6.65 $\pm$ 0.06 (9) ⁺	190 $\pm$ 9.82 (7) ⁺	197 $\pm$ 10.71 (9)
L-NAME	6.70 $\pm$ 0.05 (11) ⁺	6.53 $\pm$ 0.06 (12) ⁺	201 $\pm$ 12.28 (11) ⁺	204 $\pm$ 17.77 (12)
SOD	5.95 $\pm$ 0.13 (6)	5.97 $\pm$ 0.13 (6)	166 $\pm$ 12.80 (6)	164 $\pm$ 5.20 (6) ⁺
SOD plus CAT	5.75 $\pm$ 0.13 (7)	5.92 $\pm$ 0.11 (7)	180 $\pm$ 7.76 (7)	171 $\pm$ 4.87 (7) ⁺
INDO	6.01 $\pm$ 0.07 (6)	6.05 $\pm$ 0.09 (6)	170 $\pm$ 16.85 (6)	152 $\pm$ 22.54 (6) ⁺
NS-398	5.82 $\pm$ 0.14 (4)	5.69 $\pm$ 0.05 (5) ⁺	163 $\pm$ 16.23 (4)	158 $\pm$ 6.52 (5) ⁺
Ridogrel	6.02 $\pm$ 0.08 (7)	5.80 $\pm$ 0.09 (7) ⁺	176 $\pm$ 7.61 (7)	159 $\pm$ 9.34 (7) ⁺

Maximum response (E_{max}) and sensitivity (pD₂) to noradrenaline of endothelium denudation (E–) or intact (E+) MRA incubated with L-NAME, SOD, SOD plus catalase (CAT), indomethacin (INDO), N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide (NS-398) or ridogrel from vehicle (VHE) and ouabain (OUA)-treated rats for 20 weeks. Results are expressed as means $\pm$ SEM for the number of animals used. Number of rats is indicated in parentheses. t test: * p < 0.05 versus VHE; ⁺ p < 0.05 versus E+.

Fig. 3. Effect of endothelium denudation (E-; **a**), and L-NAME (**b**) or SOD (**c**) incubation on the contraction induced by noradrenaline in MRA from VHE and OUA-treated rats for 20 weeks. Number of rats is indicated in parentheses. Results are expressed as means $\pm$ SEM for the number of animals used.

Fig. 4. Effect of NS-398 (**a**) or ridogrel (**b**) incubation on the contraction induced by noradrenaline in MRA from VHE and OUA-treated rats for 20 weeks. Number of rats is indicated in parentheses. Results are expressed as means $\pm$ SEM for the number of animals used.

Discussion

Increasing evidence indicates that endogenous ouabain is able to modulate blood pressure [2, 3]. Various studies have demonstrated that exposure to ouabain for 2–5 weeks leads to the development of hypertension, but no studies have demonstrated a plateau in blood pressure following this treatment period [7–14, 24]. Our data show for the first time that the maximal hypertensive effect of

small doses of ouabain is reached within 6 weeks of treatment, and that this hypertension is maintained over 20 weeks of exposure to the glycoside hormone.

Ouabain-induced hypertension involves central mechanisms associated with increased sympathetic tone [16, 17] and peripheral adjustments [9–14, 18–20]. In the initial stages of hypertension induced by chronic ouabain treatment, the contractile response to phenylephrine in MRA remained unchanged [19]. In addition, Xavier et al.

Fig. 5. Effect of ouabain treatment for 20 weeks on COX-1, COX-2, IL-1β, IL-6 and TNF-α protein expression in MRA of Wistar rats. Upper panels show representative blot for COX-1 (a), COX-2 (b), IL-1β (c), IL-6 (d) and TNF-α (e) protein expression in MRA from VHE and OUA rats. Positive control for COX-1, COX-2, IL-1β, IL-6 and TNF-α protein (lungs of rats previously activated with lipopolissacaride). Lower panel shows densitometric analysis of

the Western blot for COX-1 (a), COX-2 (b), IL-1β (c), IL-6 (d) and TNF-α (e) protein expression. Results (means $\pm$ SEM) are expressed as the ratio between the signal for COX-1, COX-2, IL-1β, IL-6 and TNF-α and the signal for their corresponding α-actin. Number of animals used was between 5 and 7. t test: * $p < 0.05$ versus VHE.

[12] have also observed that 5 weeks ouabain-induced hypertension did not alter the response of MRA to nor-adrenaline. However, Hernanz et al. [18] suggested that 5 weeks ouabain-induced hypertension increased both basal NO and the relaxation induced by bradykinin and

L-arginine in cerebral arteries, without changing the vasoconstrictor response to 5-hydroxytryptamine.

In line with this evidence, we analyzed whether ouabain-induced hypertension for longer periods as 10 and 20 weeks was accompanied by alterations in vascular

Fig. 6. Effect of ouabain treatment for 20 weeks and COX-2 inhibition (NS-398) on ROS generation in MRA of Wistar rats. Upper panel shows representative fluorescence photomicrographs of microscopic sections of MRA from VHE and OUA-treated rats for 20 weeks incubated with or without NS-398. Lower panel shows densitometric analysis of fluorescence intensity of MRA from VHE and OUA-treated rats for 20 weeks incubated with or without NS-398. Number of animals used was 4. ANOVA (two way): * $p < 0.05$ versus VHE.

function, and also, whether these alterations differed from those observed in 5-week ouabain-treated rats. Our results demonstrate that the acetylcholine-induced relaxation was unaffected by 10 and 20 weeks of ouabain treatment, in a similar manner to vessels from 5-week ouabain-treated rats as observed by Xavier et al. [12] and confirmed in the present study. However, constriction to noradrenaline was altered in a time-dependent manner. Thus, while 5 and 10 weeks of treatment did not alter this response, after 20 weeks of ouabain treatment the noradrenaline-induced contraction in MRA was significantly enhanced. In contrast to the present results and previous studies [14] using third-order MRA from 5-week ouabain-treated rats, Zhang et al. [25] demonstrated that fourth-order MRA from these animals exhibited augmented responses to high-dose phenylephrine. This divergence between studies may be due to the differences in the branches from mesenteric artery assessed, although in both studies the mesenteric arteries evaluated (feeder or more distal arteries) are truly resistance arter-

ies, as characterized by Christensen and Mulvany [26]. Therefore, the present results suggest that longer ouabain administration enhances the vascular reactivity from MRA, which in turn may collaborate with the increased blood pressure observed in these animals.

As the endothelium plays a crucial role in the vascular effects of ouabain-induced hypertension [9, 10, 12, 18], we investigated the participation of endothelium-derived factors in the constriction to noradrenaline of resistance arteries only from 20-week ouabain-treated rats, since no differences were observed in vascular function in shorter periods of treatment (5 and 10 weeks). Our results revealed that endothelium removal and NO synthase inhibition produced smaller increases in constriction to noradrenaline in arteries from 20-week ouabain-treated rats compared to VHE-treated rats. The minor role of NO as a vasodilator may be associated with decreased synthesis and/or bioavailability. In the current study, Western blot analysis demonstrated no change in eNOS protein expression in MRA. In line with these results, other inves-

tigators have demonstrated that 5 weeks of ouabain treatment did not affect eNOS protein expression in MRA or cerebral arteries [12, 18]. As eNOS protein expression remained unchanged after 20 weeks of ouabain treatment, the loss of the vasodilator effect of NO seems to be related to its degradation.

Under certain circumstances such as hypertension, degradation of NO is increased in the presence of excessive levels of superoxide anions (O_2^-) [27, 28], which can lead to an increased vasoconstrictor response. Aizman et al. [29] have demonstrated that low concentrations of ouabain induce cross-talk inside the cell, involving highly complex signaling cascades that lead to the formation of ROS. In line with these results, Valente et al. [30] identified O_2^- as one of the ROS formed by ouabain treatment in Madin-Darby canine kidney epithelial cells. In the present study, incubation with SOD decreased noradrenaline-induced contraction in resistance arteries from 20-week ouabain-treated rats, indicating a role for O_2^- in the augmented noradrenaline-induced contraction observed in these vessels. As SOD plus catalase did not have further effects on the response observed with SOD alone, alterations in hydrogen peroxide levels do not seem to be involved in the effects of chronic ouabain treatment. In addition, we were able to demonstrate an increase in ROS generation through oxidation of DHE in arteries from 20-week ouabain-treated rats. As an increase in O_2^- concentration can result in enhanced inactivation of NO [28], these results suggest that the increased degradation of NO by O_2^- contributes to the augmented noradrenaline response in MRA from ouabain-treated rats by 20 weeks.

Endothelial cells can also release metabolites of arachidonic acid, which are thought to contribute to the α -adrenergic contractile response in hypertensive rats [31, 32]. Activation of thromboxane receptors allows the production of vasoconstrictor prostanoids such as prostaglandin H_2 and/or thromboxane A_2 , which are also thought to participate in the endothelial dysfunction associated with different cardiovascular risk factors [31, 33–35]. It has been reported that ouabain may induce the release of prostanoids from the vascular endothelium [36]. Moreover, Padilha et al. [19] have demonstrated that in the initial stages of chronic ouabain-induced hypertension, COX-2-derived prostanoids are involved in the modulation of the phenylephrine response in MRA. In the present study, both indomethacin, a COX-1/2 inhibitor, and NS-398, a specific COX-2 inhibitor, were able to reduce the noradrenaline response, suggesting a role for COX-2-derived prostanoids in the augmented response to noradrenaline in vessels from 20-week ouabain-treated

rats. In support of these results, protein expression of COX-2, but not COX-1, was increased in MRA from this group. Furthermore, incubation with ridogrel also decreased the contractile response to noradrenaline in resistance arteries from 20-week ouabain-treated rats, suggesting that thromboxane A_2 and/or prostaglandin H_2 are the major vasoconstrictor prostanoids stimulated by noradrenaline in MRA. Consistent with these results, our group has demonstrated that in spontaneously hypertensive rats treated with ouabain for 5 weeks, the response to phenylephrine in small arteries is increased and it is mainly modulated by thromboxane A_2 and/or prostaglandin H_2 derived from COX-2 metabolism [21].

It is now clear that COX-2 may also generate O_2^- in some circumstances [37, 38]. As pointed out above, the oxidative stress and COX-2 pathways are involved in the vascular damage from ouabain-treated rats. Considering these facts, we evaluated whether COX-2 could be a potential source of O_2^- . Thus, we have shown that the incubation with the specific inhibitor of COX-2 decreased DHE fluorescence in arteries from 20-week ouabain-treated rats to levels similar to those observed in arteries from VHE rats. Taken together, our data strongly suggest, for the first time, that COX-2 is an important source of O_2^- in ouabain-induced hypertension.

There is increasing evidence to suggest that hypertension could be considered as a chronic inflammatory disease with elevated pro-inflammatory cytokines [39] and COX-derived prostanoids, mainly from the COX-2 isoform [31]. In addition, there are reports that ouabain can stimulate signaling pathways to promote activation of inflammatory factors such as cytokines [2]. The expression of enzymes such as COX-2, iNOS and NADPH oxidase is then thought to be increased by cytokines [38, 40]. Moreover, acute ouabain administration has been found to induce transcription of IL-1 β , IL-6 and TNF- α in human peripheral blood mononuclear cells [41]. In addition, Bereta et al. [42] have shown that acute ouabain administration was able to stimulate vascular cell adhesion molecule expression in murine microvascular cells and thus provide a complementary signal to either TNF- α or IFN- γ in inducing iNOS expression. Based on this evidence, and on endothelial dysfunction in MRA induced by long-term ouabain treatment, we analyzed whether 20-week ouabain-induced hypertension was accompanied by alterations in protein expression of the pro-inflammatory cytokines IL-1 β , IL-6 and TNF- α . Our results demonstrate that in MRA, the protein expression of TNF- α and IL-6 was enhanced. These findings therefore suggest that 20-week ouabain-induced hypertension is characterized

by vascular pro-inflammatory events that can contribute not only to an increased noradrenaline response, but also to advanced vascular damage in this model.

In conclusion, our findings demonstrate for the first time that chronic treatment with low doses of ouabain for 10 and 20 weeks induces a maintained mild hypertension. Nevertheless, only after 20 weeks of ouabain treatment was damage in the vascular function observed, which can be related to COX-2-derived prostanoids, oxidative stress and reduced NO bioavailability. Additionally, the injury in vascular function is associated with an increase in pro-inflammatory cytokines. Accumulating evidence from experimental and clinical trials support the notion that endogenous ouabain may play an important role in hypertension, which is associated to altera-

tions in vascular function. Therefore, it seems reasonable to assume that an antihypertensive drug able to antagonize the effects of ouabain could represent a novel therapeutic approach, as described by Ferrari et al. [43].

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