J Physiol Biochem, **64** (2), 115-126, 2008

Chronic ouabain treatment increases the contribution of nitric oxide to endothelium-dependent relaxation

R. Aras-López[1], J. Blanco-Rivero[1], R. Hernanz[2], A.M. Briones[2], L.V. Rossoni[3], M. Ferrer[1], M. Salaices[2] and G. Balfagón[1]

Departamentos de [1]Fisiología, [2]Farmacología y Terapéutica, Facultad de Medicina, Universidad Autónoma de Madrid, 28029 Madrid, Spain. [3]Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil

(Received on November, 2007)

R. ARAS-LÓPEZ, J. BLANCO-RIVERO, R. HERNANZ, A.M. BRIONES, L. V. ROSSONI, M. FERRER, M. SALAICES and G. BALFAGÓN. *Chronic ouabain treatment increases the contribution of nitric oxide to endothelium-dependent relaxation.* J Physiol Biochem, **64** (2), 115-126, 2008.

The aim of this study was to analyze the contribution of nitric oxide, prostacyclin and endothelium-dependent hyperpolarizing factor to endothelium-dependent vasodilation induced by acetylcholine in rat aorta from control and ouabain-induced hypertensive rats. Preincubation with the nitric oxide synthase inhibitor N-omega-nitro-L-arginine methyl esther (L-NAME) inhibited the vasodilator response to acetylcholine in segments from both groups but to a greater extent in segments from ouabain-treated rats. Basal and acetylcholine-induced nitric oxide release were higher in segments from ouabain-treated rats. Preincubation with the prostacyclin synthesis inhibitor tranylcypromine or with the cyclooxygenase inhibitor indomethacin inhibited the vasodilator response to acetylcholine in aortic segments from both groups. The Ca^{2+}-dependent potassium channel blocker charybdotoxin inhibited the vasodilator response to acetylcholine only in segments from control rats. These results indicate that hypertension induced by chronic ouabain treatment is accompanied by increased endothelial nitric oxide participation and impaired endothelium-dependent hyperpolarizing factor contribution in acetylcholine-induced relaxation. These effects might explain the lack of effect of ouabain treatment on acetylcholine responses in rat aorta.

Keywords: Nitric oxide, Endothelial-dependent hyperpolarizing factor, Prostacyclin, Acetylcholine.

Correspondence to G. Balfagón (Tel.: +34 91 4975450; Fax. +34 91 4975478; e-mail: gloria.balfagon@uam.es).

Ouabain, a sodium pump inhibitor, regulates the body fluid balance and urine sodium excretion and increases sympathetic tone by a central mechanism triggered by the activation of cerebral renin-angiotensin and endothelin systems (10). The role of an endogenous ouabain-like compound in the development and/or maintenance of hypertension has been suggested by findings showing that ouabain plasma levels are elevated in some forms of hypertension, including essential human hypertension (10, 24). In addition, several reports have also demonstrated that chronic administration of ouabain, or its structurally related analogs, induces hypertension in rats (22, 35). We have demonstrated that this hypertensive animal model is accompanied by alterations in the endothelial modulation of vascular contractility. In rat aorta, chronic ouabain treatment induced a decrease of the vasoconstrictor response to phenylephrine associated with increases in endothelial hyperpolarizing factor and nitric oxide release (22). In rat superior mesenteric artery, increased release of endothelial nitric oxide and decreased prostanoid participation in phenilephrine vasoconstriction was observed after ouabain treatment (32). However, vasoconstrictor responses to alpha-adrenoceptor agonists did not change in mesenteric resistance arteries in this hypertensive model probably due to enhanced endothelial nitric oxide release and impaired contribution of the endothelium-dependent hyperpolarizing factor (32). Collectively, these observations indicate that chronic treatment with ouabain alters the contribution of endothelial factors to vasoconstrictor responses, which may be associated with changes in such responses in the different vascular beds.

Endothelium-dependent dilatation to acetylcholine is modulated by three principal mediators: nitric oxide, cyclooxygenase-derived prostanoids, specially prostacyclin and the still unidentified endothelium-derived hyperpolarizing factor (6, 25). The vasodilator responses to nitric oxide and prostacyclin are mediated by activation of cGMP and cAMP respectively, while the effect of endothelium-derived hyperpolarizing factor is through opening of large-conductance Ca^{2+}-activated potassium channel (7). On the other hand, nitric oxide would induce hyperpolarization of smooth muscle through the opening of large-conductance Ca^{2+}-activated potassium channels (4).

Endothelial dysfunction, usually associated with vascular diseases such as hypertension (27), is reported to be associated to 1) decreased nitric oxide synthesis and/or increased of its metabolism (14), 2) increased production of contractile cyclooxygenase-derived prostanoids, 3) decreased prostacyclin synthesis (16) and 4) altered endothelium-dependent hyperpolarizing factor pathway (7). In previous studies, we have found no changes in the vasodilator response to acetylcholine in conductance and resistance arteries from ouabain-induced hypertension model (21, 32). Although the endothelium dependent vasodilator response did not change, the contribution of the different endothelial factors to vasoconstrictor response does change in this hypertensive model. Thus, the aim of this study was to analyze the contribution of nitric oxide, prostacyclin and endothelium-dependent hyperpolarizing factor to endothelium-dependent vasodilation induced by acetylcholine in rat aorta from control and ouabain-induced hypertensive rats.

Material and Methods

Tissue preparations.– Six-week-old male Wistar rats were obtained from colonies maintained at the Animals Quarters of the Facultad de Medicina of the Universidad Autónoma de Madrid (Registration No. EX-021U). During treatment, rats were housed at a constant room temperature, humidity and light cycle (12-h light/dark) and had free access to tap water and standard rat chow. All animals were housed according to directives 609/86 CEE and R.D. 233/88 of the Ministerio de Agricultura, Pesca y Alimentación of Spain.

Pellet implantation.– With the rats under anaesthesia, a small incision was made on the back of the neck, and one controlled time-release pellet containing either ouabain (0.5 mg/pellet) or vehicle, was implanted subcutaneously according to the described method (12). These pellets are designed to release a constant amount of either ouabain (≈8.0 µg/day) or vehicle for a 60-day period. Indirect systolic blood pressure was measured once a week for 5 weeks by tail-cuff plethysmography.

Reactivity experiments.– After 5-weeks treatment, rats were decapitated and the thoracic aorta was carefully dissected out, cleaned of connective tissue and placed in Krebs-Henseleit solution (KHS) at 4º C.

The method used for isometric tension recording has been described in full elsewhere (18). Briefly, two parallel stainless steel pins were introduced through the lumen of the vascular segments; one was fixed to the bath wall and the other connected to a force transducer (Grass FTO3C; Quincy, MA, USA); this was connected in turn to a model 7D Grass polygraph. Segments of thoracic aorta (4 mm in length) were suspended in an organ bath containing 5 ml KHS at 37 ºC continuosly bubbled with a 95% O_2-5% CO_2 mixture (pH of 7.4) and subjected to a tension of 1 g which was readjusted every 15 min during a 90 min equilibration period before drug administration.

Each segment was exposed to 75 mM KCl to check its functional integrity. The contraction to 75 mM KCl was similar in segments from both experimental groups [control: 2137±110 mg; ouabain-treated: 2419±137 mg; P> 0.05]. After a washout period, segments were precontracted with noradrenaline to achieve a contractile response of 50-70% of the contraction elicited by 75 mM KCl [control: 1238±68 mg; ouabain-treated: 1409 ± 123 mg; P> 0.05]. The presence of endothelium was tested by the ability of 10 µM acetylcholine to relax segments precontracted with noradrenaline. A relaxation equal to or greater than 95% was considered evidence of the functional integrity of the endothelium. Afterwards, concentration-response curves to acetylcholine (0.01 nM-100 µM) were made in arterial segments from both groups and precontracted with noradrenaline. The ability of sodium nitroprusside (0.1 nM-10 µM) and prostacyclin (0.1 nM-1 µM) to induce relaxation was also analyzed in some noradrenaline contracted segments from both groups.

The effects of the nonselective NOS inhibitor N-nitro-L-arginine methyl ester (L-NAME, 100 µM), the cyclooxygenase inhibitor indomethacin (10 µM), the prostacyclin synthesis inhibitor tranylcypromine (10 µM) and the Ca^{2+}-dependent K^+ channel blocker charybdotoxin (0.1 µM) on the concentration-response curves to acetylcholine were analyzed. These drugs were added 30 min before

generating the concentration-response curves.

Nitric oxide release.– Segments of thoracic aorta from control and ouabain-treated were subjected to a resting tension of 1 g, as indicated for the reactivity experiments. After an equilibration period of 60 min in HEPES buffer (in mM: NaCl 119; HEPES 20; $CaCl_2$ 1.2; KCl 4.6; MgSO4 1; KH_2PO_4 0.4; $NaHCO_3$ 5; glucose 5.5; Na_2HPO_4 0.15; pH 7.4) at 37 ºC, segments were incubated with the fluorescent probe 4,5-diaminofluorescein 0.5 µM for 45 min. Then, the medium was collected to measure the basal nitric oxide release. Once the organ bath was refilled, a concentration-response curve to acetylcholine (0.01 nM-100 µM) was applied to evoke nitric oxide release. The stimulation-induced nitric oxide release was calculated by subtracting basal nitric oxide release from that evoked by acetylcholine. Blank measures were collected in the same way but without segments in order to subtract background emission. The fluorescence of the medium was measured at room temperature using a spectrofluorimeter (LS50 Perkin Elmer instruments, FL WINLAB Software) with excitation wavelength set at 495 nm and emission wavelength at 515 nm. Some experiments were performed in presence of 100 µM L-NAME to ensure de specifity of the method. The amount of the nitric oxide release was expressed as arbitrary units (A.U.) per mg tissue.

Solutions, drugs and statistical analysis.– The composition of KHS was in mM: NaCl 115, $CaCl_2$ 2.5, KCl 4.6, KH_2PO_4 1.2, $MgSO_4$ 7-H_2O 1.2, $NaHCO_3$ 25, glucose 11.1, Na_2-EDTA 0.03.

Drugs used were: noradrenaline hydrochloride, acetylcholine chloride, L-NAME hydrochloride, indomethacin, tranylcypromine, prostacyclin and charybdotoxin (Sigma, St. Louis, MO, USA). Ouabain pellets (Innovative Research of America, Saratoga, FL, USA). Stock solutions (10 mM) of drugs were made in distilled water, except for noradrenaline, which was dissolved in a NaCl (0.9%)–ascorbic acid (0.01% w/v) solution, and indomethacin and prostacyclin, which were solubilized in ethanol and administered from a prepared stock in such a way that the maximal ethanol concentration of the medium was less than 0.001% (vol/vol). These solutions were kept at –20 °C and appropriate dilutions were made in KHS on the day of the experiment.

Results are expressed as mean ± SEM of the number of rats indicated. Differences were analyzed using Student's *t*-test for nitric oxide release experiments or two-way ANOVA for the relaxation-response curves to acetylcholine, using the Graphpad Prism 3.0 software (CA, USA). Some results were expressed as differences of area under the concentration-response curves (dAUC) to acetylcholine obtained in segments from control and treated animals. AUC were calculated from the individual concentration-response curves/plot and the differences were expressed as a percentage of the difference in AUC of the corresponding control situation. Differences were considered statistically significant at $P < 0.05$.

Results

After 5 weeks, ouabain-treated rats (n = 24) showed significant hypertension compared with the control group (n =18) systolic blood pressure (155 ± 2.4 mm Hg *vs.* 124 ± 2 mm Hg; $P < 0.05$) without differences in body weight gain.

In segments precontracted with noradrenaline, ouabain treatment did not modify the vasodilator response induced by acetylcholine (0.01 nM- 100 µM) (Fig. 1), sodium nitroprusside (0.1 nM-10 µM) or prostacyclin (0.1 nM-1 µM) (data not shown).

The non-specific NOS inhibitor L-NAME (100 µM) inhibited the vasodilator response to acetylcholine in segments from both groups of rats. This decrease was higher in segments from ouabain-treated rats than in control rats (Fig. 1). Basal nitric oxide release was increased in segments from ouabain-treated rats. Acetylcholine significantly increased nitric oxide release in segments from both groups; this increase was greater in segments from ouabain-treated rats (Fig. 2). Preincubation with 100 µM L-NAME abolished NO release in arteries from both groups (data not shown).

The cyclooxygenase inhibitor indomethacin (10 µM) and the prostacyclin synthesis inhibitor tranylcypromine (10 µM) similarly inhibited the vasodilator response to acetylcholine in aortic segments from ouabain-treated and control rats (Fig. 3).

The Ca^{2+}-dependent potassium channel blocker charybdotoxin (0.1 µM), inhibited the vasodilator response to acetylcholine only in segments from control rats (Fig. 4).

Incubation with L-NAME, indomethacin or charybdotoxin did not modi-

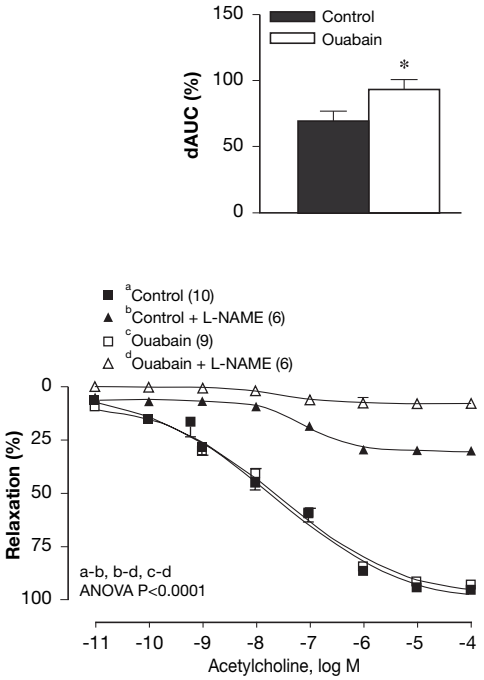

Fig. 1. *Effect of 100 µM L-NAME on the concentration-dependent relaxation to acetylcholine in aortic segments from control and ouabain-treated Wistar rats.*

Results (mean ± SEM) are expressed as a percent of a previous contraction to noradrenaline. ANOVA (two-way): $P<0.0001$. The number of animals used is indicated within parentheses. The inset graph shows the dAUC (differences of area under the curves) to acetylcholine in segments in the absence or presence of L-NAME. dAUC values (mean ± SEM) are expressed as a percentage of the difference with the corresponding AUC for segments in the absence of L-NAME (unpaired *t*-test $^{*}P<0.05$)

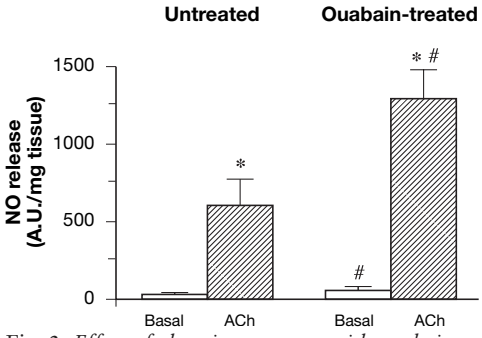

Fig. 2. *Effect of chronic treatment with ouabain on nitric oxide release in basal conditions or after acetylcholine stimulation in aortic segments from Wistar rats.*

Results (mean ± SEM) are expressed as arbitrary units (A.U.) per mg tissue. Paired *t*-test $^{*}P < 0.05$ *vs* the respective basal nitric oxide release and $^{\#}P < 0.05$ *vs* basal and after acetylcholine stimulation nitric oxide release from control rats. Number of animals within parentheses.

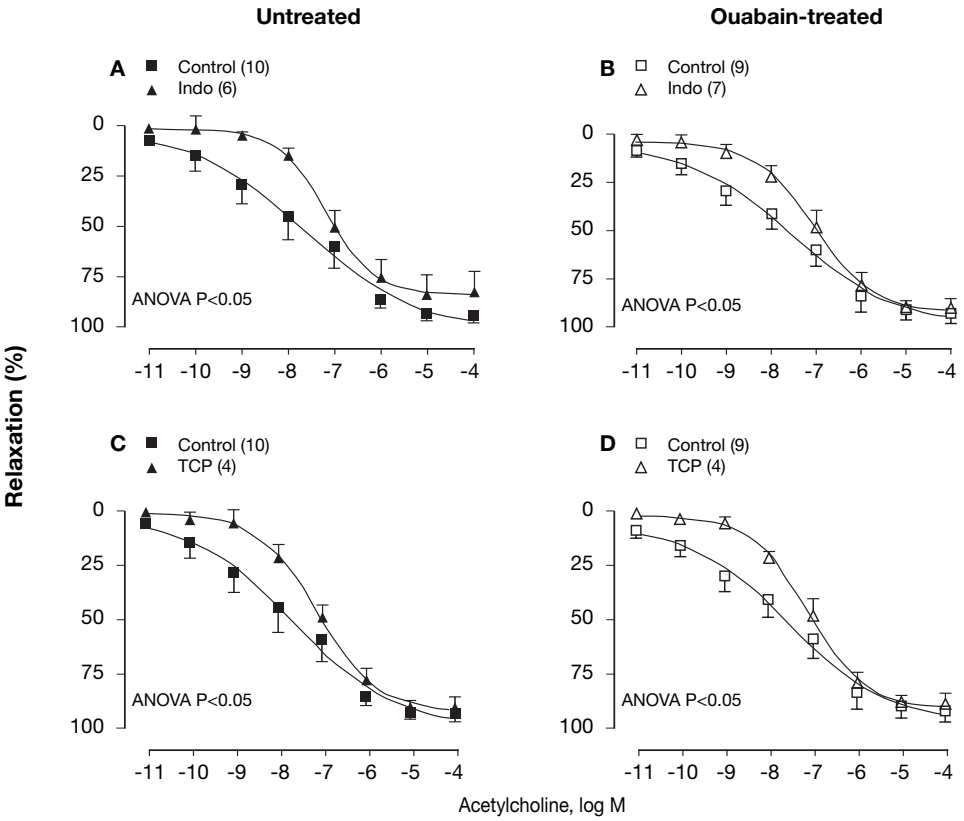

Fig. 3. *Effect of 10 μM indomethacin (Indo) (A and B) 10 μM tranylcypromine (TCP) (C and D) on the concentration-dependent relaxation to acetylcholine in aortic segments from control (A and C) and ouabain-treated (B and D) Wistar rats.*

Results (mean ± SEM) are expressed as a percent of the previous contraction to noradrenaline**.** ANOVA (two-way): $P<0.05$. Number of animals within parentheses.

fy the basal tone in segments from either control or ouabain-treated rats. However, tranylcypromine induced a sustained increase in tension that was similar in control (326.8 ± 87.82 mg) and ouabain-treated rats (405±178.4 mg).

Discussion

It is now established that chronic ouabain treatment induces hypertension in rats (9, 12, 22). This hypertension seems to depend, at least in part, on central mechanisms associated with increased sympathetic tone subsequent to the activation of renin-angiotensin (35) and endothelin (9) systems. However, additional peripheral mechanisms might occur in the hypertension induced by chronic treatment with ouabain. This hypertensive model is accompanied by a reduction in vascular reactivity to phenylephrine in aorta and superior mesenteric artery (21,

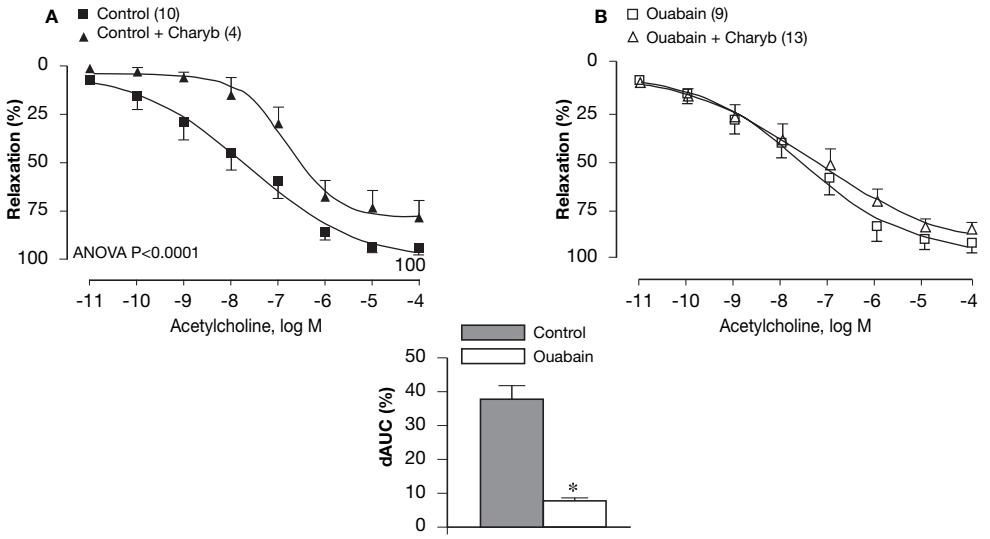

Fig. 4. *Effect of 0.1 μM charybdotoxin (Charyb) on the concentration-dependent relaxation to acetylcholine in aortic segments from control (A) and ouabain-treated (B) Wistar rats.*

Results (mean ± SEM) are expressed as a percent of the previous contraction to noradrenaline. ANOVA (two-way): $P<0.0001$. Number of animals in parentheses. The inset graph shows the dAUC to acetylcholine in segments in the absence or presence of charybdotoxin. dAUC values are expressed as a percentage of the difference of the corresponding AUC for segments in the absence of charybdotoxin (unpaired *t*-test $^*P<0.05$).

22, 32) although the alpha adrenergic responses do not change in mesenteric resistance arteries (32) or even increase in renal arteries (13). Endothelial cells induce vasodilation and modulate the contractile responses by releasing vasoactive factors including nitric oxide, endothelium-derived hyperpolarizing factor and products of the cyclooxygenase pathway. We have demonstrated that this hypertensive model induces alterations in the endothelial modulation of vasoconstrictor responses with changes in the participation of the endothelial factors (21, 22, 32). The present study was designed to analyze the effect of hypertension induced by chronic treatment with ouabain on the relative contribution of the different endothelial factors in the endothelium-dependent relaxation induced by acetylcholine.

The existence of endothelial dysfunction has been widely described in different models of hypertension. This dysfunction presents different characteristics depending of the hypertension model studied (3, 5, 27). However, the relaxation induced by acetylcholine in rat aorta remains unmodified after ouabain treatment. These results are in agreement with those previously obtained in conductance and resistance arteries (21, 32) and apparently discount the existence of endothelial alterations in this hypertensive model. The modulation of phenylephrine responses by endothelial nitric oxide and the expression of eNOS in rat aorta was greatly augmented after ouabain treatment (22). In addition, increases of eNOS activity and expression have been associated to hypertension (29). It has been described that endothelium dependent relaxation to

acetylcholine in rat aorta is mainly mediated by the release of nitric oxide from the endothelium (31) although other endothelial factors could also be implicated (8). In order to know whether the participation of endothelial nitric oxide in acetylcholine relaxation is altered with ouabain treatment we studied the effect of the NOS inhibitor, L-NAME on acetylcholine relaxation. This inhibitor reduced acetylcholine relaxation in rat aorta from both groups, but to a greater extent in segments from ouabain-treated rats. These results indicate that the participation of endothelial nitric oxide in acetylcholine vasodilator responses is augmented in this hypertensive model obtained by ouabain administration. In this line of evidence, we have previously demonstrated that the endothelial modulation of vasoconstrictor responses by nitric oxide was greater in vessels from ouabain-treated rats (22, 32). This could be a compensatory mechanism consequence of a direct vascular action by ouabain and/ or an adaptive response to the increased sympathetic outflow. The greater participation of nitric oxide in vasodilator responses to acetylcholine was not due to an alteration in the response to nitric oxide since sodium nitroprusside responses were similar in aorta from both groups. The quantification of nitric oxide release induced by acetylcholine, measured as fluorescence emitted by 4,5-diaminofluorescein, showed higher values in segments from ouabain-treated rats suggesting that a greater synthesis of nitric oxide in these segments would be responsible for the increased participation of nitric oxide in the acetylcholine responses. In agreement, acute ouabain incubation increased nitric oxide release in carotid arteries (33) and in smooth muscle cells (19).

Prostacyclin is another endothelial factor that participates in the endothelium dependent vasodilator response induced by acetylcholine (8). Earlier studies reported that ouabain induces prostacyclin release from bovine endothelial cells (15) and enhances its production in aortic endothelial cells stimulated by ATP or bradykinin (2). In addition, alterations in the synthesis of prostaglandins in hypertension have also been reported (27, 28). In the present study, both indomethacin and the prostacyclin synthesis inhibitor tranylcypromine induced a small but significant reduction of the vasodilator response induced by low concentrations of acetylcholine in segments from both groups of rats, indicating the participation of prostacyclin in the vasodilator response induced by acetylcholine at low concentrations. Some investigators have also reported that prostacyclin plays a secondary role in the relaxation induced by acetylcholine in rat aorta (9) although other investigators have not found any effect of COX inhibitor in the vasodilator responses induced by acetylcholine in this vessel (1). The reason for this discrepancy may lie in the higher acetylcholine concentration used in those studies. The effect of indomethacin and tranylcypromine on acetylcholine responses was similar in both control and ouabain-treated groups in our study. Moreover, the vasodilator responses to exogenous prostacyclin remained unaffected by ouabain treatment. These results suggest that ouabain-induced hypertension is not accompanied by changes in the synthesis or effect of vasodilator prostanoids in this artery.

In addition to nitric oxide and prostacyclin, endothelium-derived hyperpolarizing factor appears to be a mediator of acetylcholine-induced relaxation in different vascular beds (11). The various candi-

dates suggested to explain endothelium-derived hyperpolarizing factor-mediated responses in blood vessels include epoxyeicoatetranoic acid derived from the cytochrome P450 monooxigenase, potassium ion and hydrogen peroxide (7). Endothelium-derived hyperpolarizing factor is known to induce hyperpolarization and vasodilatation through activation of potassium channels (small- and intermediate-conductance Ca^{2+}-activated potassium channels in endothelial cells and large-conductance Ca^{2+}-activated potassium channels in smooth muscle cells) and the Na^+/K^+ ATPase pump (7). Some studies have described an inhibition of potassium channels induced by ouabain (23). In addition, nanomolar concentrations of ouabain induce the release of an endothelium-derived relaxing factor that seems to open intermediate-conductance Ca^{2+}-activated potassium channels in the rat-tail arterial bed preparation (20). In our study, the functional contribution of endothelium-derived hyperpolarizing factor in aortas from ouabain-treated and control rats was assessed with charibdotoxin which blocks intermediate-, large-conductance Ca^{2+}-activated potassium channels as well as some voltage-dependent potassium channels (7). The vasodilator responses to acetylcholine were reduced by charibdotoxin only in aorta from control rats. This suggests a decreased participation of some factor that opens potassium channels and would elicit hyperpolarization in segments from ouabain hypertensive animals. Increased (26) and decreased (30) endothelium-derived hyperpolarizing factor production have been both described in different hypertension models. In the ouabain-induced hypertension model, we have reported an increase (22) and decrease (32) of endothelium-derived hyperpolarizing factor participation in rat aorta and mesenteric resistance arteries respectively. As acetylcholine-induced relaxation was similar in both control and ouabain-treated rats, we suggest that the increased NO production could compensate the lack of endothelium derived hyperpolarizing factor participation in ouabain-treated rats, in order to maintain a normal endothelium-dependent function.

In conclusion, our results suggest that hypertension induced by chronic ouabain treatment is accompanied by an increase in the release of endothelial nitric oxide and an impairment of the effects of an endothelium-dependent hyperpolarizing factor; both effects might explain the lack of effect of ouabain treatment on acetylcholine responses in rat aorta.

Acknowledgements

We thank to veterinarian, Dra. M.C. Fernández-Criado, for the care of the animals, and V. Sánchez and A. Pastor for their technical assistance. This work was supported by grants from Instituto de Salud Carlos III (PI051767 and RD06/0014/0011), Ministerio de Educación y Ciencia (SAF2006-02376; SAF2006-07888), Fundación Médica Mutua Madrileña (MMA-06) and Banco de Santander Central Hispano.

R. ARAS-LÓPEZ, J. BLANCO-RIVERO, R. HERNANZ, A.M. BRIONES, L. V. ROSSONI, M. FERRER, M. SALAICES y G. BALFAGÓN. *El tratamiento crónico con ouabaína incrementa la contribución del óxido nítrico a la relajación dependiente de endotelio.* J Physiol Biochem, **64** (2), 115-126, 2008.

Se analiza en este trabajo la contribución del óxido nítrico, la prostaciclina y el factor hiperpolarizante dependiente de endotelio (EDHF) en la vasodilatación inducida por acetilcolina en aorta de ratas controles y con hipertensión inducida por ouabaína. La preincubación con el inhibidor de la óxido nítrico sintasa N-omega-nitro-L-arginina metil éster (L-NAME) inhibió la respuesta vasodilatadora a acetilcolina en segmentos de ambos grupos

experimentales, pero en mayor medida en los de ratas tratadas con ouabaína. La liberación de óxido nítrico basal e inducida por acetilcolina fue mayor en segmentos de animales tratados con ouabaína. La preincubación con tranilcipromina, inhibidor de la prostaciclina sintasa, y con indometacina, inhibidor de la ciclooxigenasa, inhibió la respuesta vasodilatadora a acetilcolina en segmentos de aorta de ambos grupos experimentales. El bloqueante de los canales de potasio dependientes de Ca^{2+}, charibdotoxina, inhibió la respuesta vasodilatadora a acetilcolina sólo en segmentos de animales control. Estos resultados indican que la hipertensión inducida por el tratamiento crónico con ouabaína se acompaña de aumento en la participación del óxido nítrico endotelial y de disminución del efecto del EDHF mediado por canales de potasio dependientes de Ca^{2+} en la relajación inducida por acetilcolina. Estos efectos opuestos podrían explicar el hecho de que el tratamiento con ouabaína no modifique la respuesta a la acetilcolina.

Palabras clave: Óxido nítrico, Factor hiperpolarizante dependiente de endotelio, Prostaciclina, Acetilcolina.

References

1. Álvarez de Sotomayor, M., Pérez-Guerrero, C., Herrera, MD. and Marhuenda, E. (1999): Effects of chronic treatment with simvastatin on endothelial dysfunction in spontaneously hypertensive rats. *J Hypertens,***17**, 769-776.
2. Boeynaems, JM, Ramboer, I. (1989): Effects of changes in extra- and intracellular K+ on the endothelial production of prostacyclin. *Br J Pharmacol,* **98**, 966-972.
3. Bolad, I. and Delafontaine, P. (2005): Endothelial dysfunction: its role in hypertensive coronary disease. *Curr Opin Cardiol*, **20**, 270-274.
4. Bolotina, V.M., Najibi, S., Palacino, J.J., Pagano, P.J., Cohen, R.A. (1994): Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature*, **368**, 850-853.
5. Boulanger, C.M. (1999): Secondary endothelial dysfunction: hypertension and heart failure. *J Mol Cell Cardiol*, **31**, 39-49.
6. Bryan, R.M., Jr., You, J., Golding, E.M. and Marrelli, S.P. (2005): Endothelium-derived hyperpolarizing factor: a cousin to nitric oxide and prostacyclin. *Anesthesiology*, **102**, 1261-1277.
7. Busse, R., Edwards, G., Féletou, M., Fleming, I., Vanhoutte, P.M. and Weston, A.H. (2002): EDHF: bringing the concepts together. *Trends Pharmacol Sci*, **23**, 374-380.
8. Callera, G.E., Varanda, W.A. and Bendhack, L.M. (2000): Impaired relaxation to acetylcholine in 2K-1C hypertensive rat aortas involves changes in membrane hyperpolarization insted of an abnormal contribution of endothelial factors. *Gen Pharmacol*, **34**, 379-389.
9. Di Filippo, C., Filippelli, A., Rinaldi, B., Fiegari, E., Esposito, F., Rossi, F. and D'amico, M. (2003): Chronic peripheral ouabain treatment affects the brain endothelin system of rats. *J Hypertens*, **21**, 747-753.
10. Ferrandi, M., Manunta, P., Ferrari, P. and Bianchi, G. (2005): The endogenous ouabain: molecular basis of its role in hypertension and cardiovascular complications. *Front Biosci,* **10**, 2472-2477.
11. Garland, C.J., Plane, F., Kemp, B.K., Cocks, T.M. (1995): Endothelium-dependent hyperpolarization: a role in the control of vascular tone. *Trends Pharmacol Sci,* **16**, 23-30.
12. Huang, B.S., Huang, X., Harmsen, E. and Leenin, F.H.H. (1994): Chronic central *versus* peripheral ouabain, blood pressure, and sympathetic activity in rats. *Hypertension*, **23**, 1087-1090.
13. Kimura, K., Manunta, P., Hamilton, B.P. and 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.
14. Munzel, T., Daiber, A., Ullrich, V., and Mulsch, A. (2005): Vascular consequences of endothelial nitric oxide synthase uncoupling for the activity and expression of the soluble guanylyl cyclase and the cGMP-dependent protein kinase. *Arterioscler Thromb Vasc Biol,* **25**, 1551-1557.
15. Nagakawa, M., Takamatsu, H., Toyoda, T., Sawada, S., Tsuji, Ha. and Ijichi, H. (1987): Effect of inhibition of Na^+-K^+-ATPase on the prostacyclin generation of cultured human vascular endothelial cells. *Life Sci,* **40**, 351-357.
16. Nakayama, T. (2005): Prostacyclin synthase gene: genetic polymorphisms and prevention of some cardiovascular diseases. *Curr Med Chem Cardiovasc Hematol Agents,* 3(2), 157-164.
17. Nava, E., Noll, G. and Lüscher, T.F. (1995): Increased activity of constitutive nitric oxide

synthase in cardiac endothelium in spontaneous hypertension. *Circulation*, **91**, 2310-2313.
18. Nielsen, K.C., Owman, C. (1971): Contractile response and amine receptor mechanisms in isolated middle cerebral artery of the cat. *Brain Res* **27**, 33-42.
19. Pacheco, M.E., Marín, J., Manso, A.M., Rodríguez-Martínez, M.A., Briones, A., Salaices, M. and Redondo, J. (2000): Nitric oxide synthase induction by ouabain in vascular smooth muscle cells from normotensive and hypertensive rats. *J Hypertens*, **18**, 877-884.
20. Rossoni, L.V., Cunha, V., Franca, 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.
21. Rossoni, L.V., Salaices, M., Marín, J., Vassallo, D.V. and Alonso, M.J. (2002): Alterations in phenylephrine-induced contractions and the vascular expression of Na^+, K^+-ATPase in ouabain-induced hypertension. *Br J Pharmacol*, **135**, 771-781.
22. Rossoni, L.V., Salaices, M., Miguel, M., Briones, A.M., Barker, L.A., Vasallo, D.V. and Alonso, M.J. (2002). Ouabain-induced hypertension is accompanied by increases in endothelial vasodilator factors. *Am J Physiol Heart Circ Physiol*, **283**, H2110-H2118.
23. Saxena, N.C., Fan, J.S. and Tseng, G.N. (1997): Effects of elevating [Na]i on membrane currents of canine ventricular myocytes: role of intracellular Ca ions. *Cardiovasc Res*, **33**, 548-560.
24. Schoner, W. and Scheiner-Bobis, G. (2005): Endogenous cardiac glycosides: hormones using the sodium pump as signal transducer. *Semin Nephrol*, **25**, 343-351.
25. Shimokawa, H. and Takeshita, A. (1995): Endothelium-dependent regulation of the cardiovascular system. *Intern Med*, **34**, 939-946.
26. Taddei, S., Ghiadoni, L., Virdis, A., Buralli, S. and Salvetti, A. (1999): Vasodilatation to bradykinin is mediated by an ouabain-sensitive pathway as a compensatory mechanism for impaired nitric oxide availability in essential hypertensive rats. *Circulation*, **100**, 1400-1405.
27. Thuillez, C. and Richard, V. (2005): Targeting endothelial dysfunction in hypertensive subjects. *J Hum Hypertens*, **19**, S21-S25.
28. Vanhoutte, P.M. (1996): Endothelial dysfunction in hypertension. *J Hypertens*, **14**, S83-S93.
29. Vaziri, N.D., Ni, Z. and Oveisi, F. (1998): Upregulation of renal and vascular nitric oxide synthase in young spontaneously hypertensive rats. *Hypertension*, **31**, 1248-1254.
30. Vázquez-Pérez, S., Navarro-Cid, J., De Las Heras, N., Cediel, E., Sanz-Rosa, D., Ruilope, L.M., Cachofeiro, V. and Lahera, V. (2001): Relevance of endothelium-derived hyperpolarizing factor in the effects of hypertension on rat coronary relaxations. *J Hypertens*, **19**, 539-545.
31. Woodman, O.L. and Boujaoude, M. (2004): Chronic treatment of male rats with daidzein and 17β-oestradiol induces the contribution of EDHF to endothelium-dependent relaxation. *Br J Pharmacol*, **41**, 322-328.
32. Xavier, F.E., Rossoni, L.V., Alonso, M.J., Balfagon, G., Vassallo, D.V. and Salaices, M. (2004): Ouabain-induced hypertension alters the participation of endothelial factor in α-adrenergic responses differently in rat resistance and conductance mesenteric arteries. *Br J Pharmacol*, **143**, 215-225.
33. Xie, J., Wang, Y., Summer, W.R. and Greenberg, S.S. (1993): Ouabain enhances basal release of nitric oxide from carotid artery. *Am J Med Sci*, **305**, 157-163.
34. Yuan, C., Manunta, P., Chen, S., Hamlyn, J.M., Haddy, F.J. and Pamnani, M.V. (1993): Role of ouabain-like factors in hypertension: effects of ouabain and certain endogenous oaubain-like factors in hypertension. *J Cardiovasc Pharmacol*, **22**, S10-S12.
35. Zhang, J. and Leenen, F.H. (2001): AT(1) receptor blockers prevent sympathetic hyperactivity and hypertension by chronic ouabain and hypertonic saline. *Am J Physiol Heart Circ Physiol*, **280**, H1318-H1323.