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Ouabain-induced hypertension enhances left ventricular contractility in rats

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Abstract

Chronic ouabain treatment produces hypertension acting on the central nervous system and at vascular levels. However, cardiac effects in this model of hypertension are still poorly understood. Hence, the effects of hypertension induced by chronic ouabain administration (~8 μg day^{-1}, s.c.) for 5 weeks on the cardiac function were studied in Wistar rats. Ouabain induces hypertension but not myocardial hypertrophy. Awake ouabain-treated rats present an increment of the left ventricular systolic pressure and of the maximum positive and negative d*P*/d*t*. Isolated papillary muscles from ouabain-treated rats present an increment in isometric force, and this effect was present even when inotropic interventions (external Ca^{2+} increment and increased heart rate) were performed. However, the sarcoplasmic reticulum activity and the SERCA-2 protein expression did not change. On the other hand, the activity of myosin ATPase increased without changes in myosin heavy chain protein expression. In addition, the expression of α_1 and α_2 isoforms of Na^+, K^+-ATPase also increased in the left ventricle from ouabain-hypertensive rats. The present results showed positive inotropic and lusitropic effects in hearts from awake ouabain-treated rats, which are associated with an increment of the isometric force development and of the activity of myosin ATPase and expression of catalytic subunits of the Na^+, K^+-ATPase.

Keywords: Ouabain; Hypertension; Papillary muscle; Na^+; K^+-ATPase; SERCA-2; Myosin ATPase

Introduction

Digitalis has been used in clinical practice for over 200 years, since Willian Withering's original description in 1978, for their positive inotropic effect. The mechanisms of action of digitalis have been under extensive investigation for nearly 50 years, yielding one of the most specific mechanisms thus far defined for any agent so extensively used. Recent observations suggest that digitalis may have additional effects on cardiac cell function in both the short and long term (Wassertrom and Aistrup, 2004) and in addition, can be fully accepted that ouabain, a digitalis compound, is an endogenous regulator of blood pressure and Na^+, K^+-ATPase activity (Hamlyn et al., 1996; Schoner and Scheiner-Bobis, 2005).

Chronic ouabain treatment produces hypertension (Huang et al., 1994; Manunta et al., 1994; Kimura et al., 2000; Rossoni et al., 2002; Xavier et al., 2004). This hypertension seems to be depending, at least in part, to an activation of the central nervous mechanisms associated with increased sympathetic tone, subsequent to the activation of the brain renin–angiotensin (Huang et al., 1994; Huang and Leenen, 1999) and endothelin (Di Filippo et al., 2003) systems. In addition, peripheral vascular mechanisms also contribute for the maintenance of hypertension in this model (Kimura et al., 2000; Rossoni et al., 2002; Di Filippo et al., 2003; Xavier et al., 2004; Briones et al., 2006). On the other hand, it is known that hypertension is associated with structural, functional and biochemical adjustments on the cardiac tissue (Swynghedauw, 1999). However, whether hypertension induced by ouabain is associated or not with

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changes of the heart function is not completely elucidated yet.

Reports have been shown that models of hypertension commonly produce a concentric cardiac hypertrophy by a pressure overload mechanism. Although resulting from several mechanisms it usually causes myocyte enlargement, collagen accumulation, changes in the activity and expression of myosin ATPase, shift of V1 to V3 myosin heavy chain (MHC) isoforms and changes in Ca^{2+} handling by reducing sarcoplasmic reticulum activity (Mercadier et al., 1981; Tanamura et al., 1993; Arai et al., 1996; Mill et al., 1998; Shorofsky et al., 1999; Swynghedauw, 1999). In consequence, the alterations in the mechanical properties of the myocardium together with the prolongation of contraction duration and depressed shortening velocity usually impair force development (Swynghedauw, 1999; Shorofsky et al., 1999).

Knowing the beneficial cardiac effects induced by digitalis on the clinical practice, in the present moment it is extremely important to acknowledge if the endogenous ouabain system is able to affect the heart function. Studies that investigated cardiac changes on long-term ouabain treatment were performed only during 2 or 4 days in isolated adult cardiomyocytes (Müller-Ehmsen et al., 2003) and papillary muscles (El-Armouche et al., 2004). Moreover, the increment in blood pressure induced by ouabain treatment does not develop within this time.

Thus, the aim of the present study was to test, in addition to the central nervous system and peripheral vascular mechanisms already described, if alterations in cardiac function might occur as an adaptive response to the ouabain-induced hypertension. In particular, we have investigated: (1) arterial and left ventricular pressures in conscious rats, (2) ventricular weights, (3) left ventricular papillary muscle contractility, (4) activity and protein expression of myosin ATPase, (5) protein expression of the Sarcoplasmic Endoplasmic Reticulum Calcium-ATPase (SERCA) and (6) α_1 and α_2 isoforms of the Na^+, K^+-ATPase. To our knowledge, this is the first time that these parameters have been investigated in hearts from this model of hypertension.

Material and methods

Animals

Six-week-old male Wistar rats were used in this study. During treatment, rats were housed at a constant room temperature, humidity and light cycles (12-h light/dark) had free access to tap water and were fed with standard rat chow ad libitum. Care and use of laboratory animals and all experiments were conducted in compliance with the guidelines for biomedical research as stated by the Brazilian Societies of Experimental Biology.

Pellet implantation

Under anesthesia with diethyl ether, a small incision was made on the back of the neck and one controlled time-release pellet (Innovative Research of America, Florida, USA) containing either ouabain (0.5 mg $pellet^{-1}$) or vehicle (placebo) was implanted subcutaneously, as described (Rossoni et al., 2002). These pellets are designed to release a constant amount of either ouabain (≈8.0 μg/day) or vehicle for a 60-day period. The length of treatment was 5 weeks. After 5 weeks of treatment the following protocols were performed.

Arterial blood pressure and left ventricular pressure measurements

Rats were anesthetized with ketamine/xylazine/acepromazine (64.9; 3.20 and 0.78 mg/kg, i.p.) and allowed to breathe room air spontaneously. A polyethylene catheter (PE50, 8 cm, filled with heparinized saline) was introduced through the right carotid artery into the left ventricle. During this procedure arterial blood pressure and left ventricle systolic (LVSP) and end diastolic pressure (LVEDP) and their first time derivatives (positive and negative, dP/dt_{max} and dP/dt_{min}, respectively) were recorded. When the polyethylene catheter was introduced into the left ventricle it was fixed and the external part of the catheter was exteriorized in the back of the neck through the subcutaneous tissue. After 24 h LVSP and LVEDP and their first time derivatives were recorded in awake animals. After that, the LV catheter was pulled out and arterial blood pressure was measured. The maintenance of diastolic blood pressure at proper values was the guarantee that the aortic valve was not damaged.

Arterial and ventricular pressures were recorded continuously (Gould Statham P23XL transducer) in an 8-channel recorder (Gould, model 5900). Heart rate and dP/dt were determined by a biotech, triggered by pulse pressure and recorded simultaneously with the other variables.

After this procedure rats received 500 units of heparin (i.p.) and after 10 min they were anesthetized with 45 mg/kg of sodium pentobarbital (i.p.), killed by exsanguination and then the hearts were removed. For analysis of the activity and protein expression of myosin ATPase and protein expression of SERCA-2 and α isoforms of the Na^+, K^+-ATPase, hearts were rapidly frozen in liquid nitrogen and kept at −80 °C until the day of analysis.

Papillary muscles

The hearts were removed rapidly after thoracotomy and perfused through the aortic stump to permit a proper selection and dissection of the left ventricle papillary muscles. The preparations were mounted in a plexiglass chamber continuously superfused with gassed (95% O_2 and 5% CO_2) Krebs bicarbonate buffer solution, at 29±1 °C. Muscles stretched to L_{max} (muscle length at which active tension is maximal) were stimulated by isolated rectangular pulses (10 to 15 V, 12 ms duration) through a pair of platinum electrodes placed along the entire extension of the muscle. The standard stimulation rate was 0.5 Hz (steady state). Recording started after 60 min to permit the beating preparation to adapt to the new environmental conditions.

The bathing solution was a modified Krebs solution with the following composition (in mM): 120 NaCl, 5.4 KCl, 1.2 $MgCl_2$ $6H_2O$, 1.25 $CaCl_2$ $2H_2O$, 2.0 $NaH_2PO_4H_2O$, 1.2 Na_2SO_4, 27 $NaHCO_3$, 11 glucose. Developed force (F) was measured with an isometric force transducer (Nihon-Kohden, TB 612T, Tokyo) and recorded on a chart recorder (Nihon-Kohden, RM-6200, Tokyo), and normalized to the muscle cross-sectional area (g/mm^2). Considering the papillary muscle as a cylinder and tissue density as 1, the cross-sectional area was calculated by dividing the muscle length at L_{max} by its weight (cross-sectional area = 0.99 ± 0.045 and 1.00 ± 0.062 g/mm^2 for vehicle and ouabain-treated rats, respectively, $P > 0.05$, t-test). To avoid the possibility of a hypoxic core we performed experiments at low temperature (29 ± 1 °C).

The following protocols were used:

1. The effects of chronic ouabain treatment on the isometric force development were compared to vehicle-treated rats under control conditions and during changes in the rate of stimulation (0.1, 0.25, 0.5, 0.75 and 1 Hz) using the protocol previously described.
2. Under steady-state conditions force was measured at different Ca^{2+} concentrations (0.62; 1.25 and 2.5 mM) in preparations from vehicle and ouabain-treated rats.
3. Post-rest potentiation was used to provide information about the function of the sarcoplasmic reticulum (SR). The force developed during the contraction of the cardiac muscle is altered in response to changes in rate and rhythm. In cardiac muscle, the contractions occurring after short pauses are potentiated. In the rat cardiac muscle post-rest contractions increase its force as the rest period increases (Mill et al., 1992). Post rest contractions depend on pause duration and on the amount of calcium stored at intracellular sites and the relative participation of the SR are more important for post rest contractions than for steady state contractions. Pause intervals of various durations (15, 30 and 60 s) were used and the results are presented as relative potentiation (the amplitude of post-rest contractions divided by steady-state contractions) to normalize the data from different preparations.

Measurements of activity of myosin ATPase

To evaluate if chronic ouabain treatment affected myosin ATPase the enzyme activity was assayed as previously reported (Moreira et al., 2003). Myosin was prepared from minced and homogenized left ventricles, extracted briefly with KCl phosphate buffer (0.3 M KCl, 0.2 M phosphate buffer, pH 6.5). After precipitation of myosin and muscle residues by 15-fold dilution with water, the muscle residue was separated by filtration using cheesecloth. This procedure filters fragments of cells including membranes. The supernatant containing the myosin was centrifuged at 33,000×g for 30 min. After decantation of the supernatant the precipitate was redissolved in 0.6 M KCl to elute myosin under high ionic strength and 1 ml of water was added for each gram of tissue to produce a new precipitation. The material was again centrifuged at 33,000×g for 30 min and the muscle residue was separated by filtration. The material was redissolved again in 14 ml of water per gram of tissue, centrifuged, and filtered as before. The precipitate was dissolved in 50 mM HEPES, pH 7.0, and 0.6 M KCl plus 50%, v/v, glycerol and placed at −20 °C. To use the stocked myosin it was diluted in water (1:12) and centrifuged at 3000 rpm for 15 min. The precipitate was resuspended in 50 mM HEPES, pH 7.0, and 0.6 M KCl, and centrifuged at 3000 rpm again. The supernatant was used.

Myosin ATPase activity was assayed by measuring Pi liberation from 1 mM ATP in the presence of 50 mM HEPES, pH 7.0, 0.6 M KCl, 5 mM $CaCl_2$, or 10 mM EGTA in a final volume of 200 µl. Under this high ionic strength and no Mg^{2+} in the medium, only myosin activity was measured and there is no significant Ca^{2+}-ATPase activity from sarcoplasmic reticulum membranes, which request high Mg^{2+} and low Ca^{2+} concentrations. The nucleotide was added to the reaction mixture and preincubated for 5 min at 30 °C. The reaction was initiated by adding the enzyme fraction (3 to 5 µg protein) to the reaction mixture. Incubation times and protein concentration were chosen in order to ensure the linearity of the reaction. The reaction was stopped by the addition of 200 µl of 10% trichloroacetic acid. Controls with addition of the enzyme preparation after addition of trichloroacetic acid were used to correct for nonenzymatic hydrolysis of the substrate. All samples were in duplicate. The enzyme activity was calculated as the difference between the activities observed in the presence of Ca^{2+} and in the presence of 10 mM EGTA. The specific activity was reported as nmol Pi released per minute per milligram of protein unless otherwise stated. The total protein content was measured using the method described by Bradford (1976).

Protein expression of α and β myosin heavy chain isoforms

The myosin fraction was homogenized in 1 ml of water and centrifuged at 10,000×g for 40 min. The pellet was resuspended in Tris–EDTA buffer (pH 8.0) and the protein concentration was measured using the method described by Bradford (1976). Afterwards, the homogenates were boiled in 1.5 M Tris (pH 7.0), 0.25% SDS, 6.0% glycerol, 0.01% 2-mecaptoethanol and 0.0015% bromophenol blue buffer for 10 min and samples (0.75 µg protein per lane) were electrophoretically separated on a 7.5% SDS-PAGE. The gels were stained with 0.03% Coomassie Blue stain and the signals on the blots were densitometrically analyzed using the Scion Image software.

Protein expression of SERCA

Left ventricles were homogenized in 3 ml extraction buffer (100 mM Trisma, pH 7.5; 10 mM EDTA; 10% sodium dodecyl sulfate (SDS); 100 mM NaF; 10 mM sodium pyrophosphate; 10 mM sodium orthovanadate; at 100 °C) for 30 s. Samples were boiled for 5 min, centrifuged and aliquots of supernatants were used for the measurement of total protein content, as described (Bradford, 1976). Proteins (100 µg) of each sample were separated using 6.5% SDS-PAGE. The proteins in the gel

were transferred to a nitrocellulose membrane. In the sequence the membranes were incubated with anti-Serca-2 antibody (Santa Cruz Biotechnology; Santa Cruz, CA, USA) at room temperature, for 4 h. Membranes were washed and incubated with anti-IgG antibody linked to horseradish peroxidase at room temperature, for 1 h. Then membranes were incubated with substrate for peroxidase and chemiluminescence enhancer (Amersham Pharmacia Biotech) for 1 min and immediately exposed to X-ray film for 1–10 min. Films were then revealed in the conventional manner.

Protein expression of α_1 and α_2 isoforms of Na^+, K^+-ATPase

Protein expression of α_1 and α_2 isoforms of Na^+, K^+-ATPase was measured using the method described by Rossoni et al. (2002). Left ventricles were homogenized in ice-cold sucrose–Tris–EDTA buffer (in mM: Tris—50, Sucrose—250, EDTA—1.0, pH 7.4). Rat kidney microsomal fractions were used as controls for the α_1 and brain microsomal fractions for the α_2 isoforms. To prepare the microsomal fractions of left ventricle, kidney and brain an initial centrifugation was made at 10,000×*g* for 10 min at 4 °C. The supernatant was centrifuged at 100,000×*g* for 60 min. The pellet, representing the microsomal fraction, was resuspended in Tris–EDTA buffer (in mM: Tris—50, EDTA—1.0, pH 7.4) and the protein concentration was measured as described by Bradford (1976). 45 μg protein for left ventricle of ouabain-treated and vehicle rats, as well as the corresponding controls (10 μg of protein each for kidney and brain homogenates per lane) and prestained molecular SDS-PAGE standards (Bio-Rad, Laboratories, Hercules, CA, U.S.A.) were electrophoretically separated on a 7.5% SDS-PAGE and then transferred to polyvinyl difluoride membranes for 2 h at 4 °C, using a Mini Trans-Blot Transfer Cell system (Bio-Rad) containing (in mM): Tris—25, glycine—250, methanol—20% and SDS—0.05%. Then the membrane was blocked for 60 min at room temperature in Tris-buffered solution (in mM: Tris—25, NaCl—137, Tween 20—0.2%, pH 7.5) with 5% powdered non-fat milk. Next, the membrane was incubated overnight at 4 °C with anti-α_1 rabbit polyclonal IgG (0.1 mg ml^{-1} dilution) or anti-α_2 rabbit polyclonal antiserum (1:5000 dilution), all purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.). After washing, the membrane was incubated for 90 min with an anti-rabbit IgG antibody conjugated to horseradish peroxidase (1:3000 dilution; Bio-Rad). The membrane was thoroughly washed and the immunocomplexes were detected using an enhanced horseradish peroxidase/luminol chemiluminescence system (ECL Plus, Amersham International plc, Little Chalfont, U.K.) and then subjected to autoradiography for either 5 min (α_1) or 10 min (α_2).

Drugs used

Pentobarbital sodium (Cristalia—Produtos Químicos Farmacêuticos Ltd., São Paulo, SP, Brazil); Heparin (Roche Q.F.S. A., Rio de Janeiro, RJ, Brazil; Sigma) and Caffeine anhydrous were purchased from Sigma Chemical Co. (St. Louis, MO, U.S. A.). All other reagents used were of analytical grade from Sigma; E. Merk (Darmstadt, Germany) or Reagen (Rio de Janeiro, RJ, Brazil).

Statistical analysis

The results are presented as mean±SEM with *N* indicating the number of observations. Values were analyzed using *t*-test or ANOVA (one- and two-way). When ANOVA revealed a significant difference, Tukey test was applied. $P<0.05$ was taken as significant. The analysis of the data and the plotting of figures were carried out using softwares GraphPad Prism™ (version 3.0, GraphPad Software, San Diego, CA, USA) and GB-STAT (version 4.0, Dynamic Microsystem Inc., Silver Spring, MD).

Results

Arterial blood pressure and left ventricular pressure in awake animals

Five weeks of ouabain treatment did not affect the body weight of the rats (ouabain: 264±4.17 vs. vehicle: 283±11.9 g; $P>0.05$) and no signs of left (ouabain: 2.18±0.05 vs. vehicle: 2.14±0.07 mg/g; $P>0.05$) or right (ouabain: 0.62±0.03 vs. vehicle: 0.58±0.02 mg/g; $P>0.05$) ventricular hypertrophy were observed.

As previously reported, in conscious ouabain-hypertensive rats systolic and diastolic arterial pressures increased compared to the vehicle group and no differences were observed in heart rate (Table 1). However, novel hemodynamic results observed in the present study are the increment of LVSP, dP/dt_{max} and dP/dt_{min} in conscious ouabain-hypertensive compared to vehicle rats and no changes on LVEDP (Table 1).

Isolated papillary muscles

Fig. 1A shows that in control conditions (rate of stimulation of 0.5 Hz and 1.25 mM $CaCl_2$) papillary muscles from ouabain-hypertensive rats developed larger forces compared to the

Table 1
Changes in systolic (SBP) and diastolic blood pressure (DBP), in heart rate (HR), in left ventricle systolic pressure (LVSP) and end diastolic pressure (LVEDP) and in positive (dP/dt_{max}) and negative first time derivatives (dP/dt_{min}) obtained from conscious vehicle ($n=10$) and ouabain-treated ($n=10$) rats

	Vehicle	Ouabain
SBP (mm Hg)	126±2.11	146±3.16*
DBP (mm Hg)	88±2.31	97±3.35*
HR (bpm)	341±8.99	354±8.88
LVSP (mm Hg)	123±1.11	142±0.84*
LVEDP (mm Hg)	7.83±1.45	7.20±0.79
dP/dt_{max} (mm Hg/s)	6617±189	7760±167*
dP/dt_{min} (mm Hg/s)	−6105±212	−7016±157*

t-Test, $P<0.05$, *vs. vehicle.

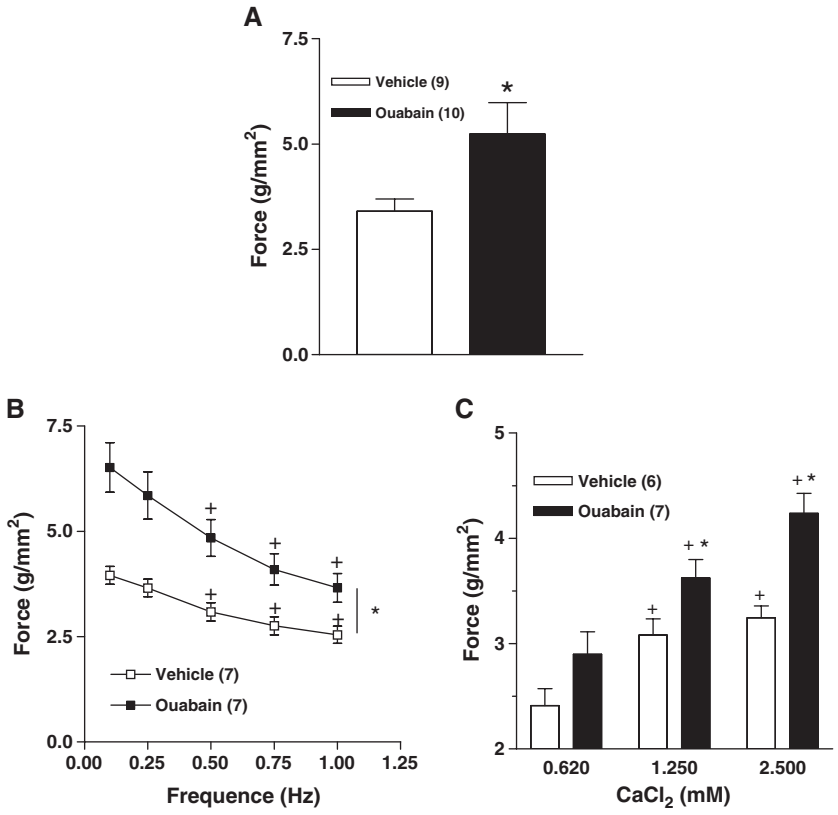

Fig. 1. (A) Comparison of developed force in control conditions; (B) under changes in rate of stimulation and (C) increasing extracellular Ca^{2+} concentration. Columns or symbols represent mean±SEM. $P<0.05$, $^{+}$vs. control conditions and *vs. vehicle.

vehicle group. To investigate if ouabain treatment affected the action of inotropic interventions, changes in heart rate and concentration-response curve to $CaCl_2$ were performed. As expected for the rat myocardium the increase of the rate of stimulation reduced force in both groups (Fig. 1B). Although force was higher in the ouabain-treated animals for all rate changes, the magnitude of force reduction was similar in both groups (magnitude of force reduction is expressed as a percentage of the response to 0.1 Hz taken this response as the maximal force development for each papillary muscle: Ouabain-treated: 57±2.99 vs. vehicle: 63±2.64% to 1 Hz rate of stimulation, $P>0.05$) (Fig. 1B). Extracellular Ca^{2+} increment increased force in both groups, and, as observed before, the ouabain-treated animals developed more force compared to vehicle group (Fig. 1C).

Following that, chronic ouabain effects on the sarcoplasmic reticulum were investigated. No differences in the relative potentiation obtained in ouabain-hypertensive and vehicle-

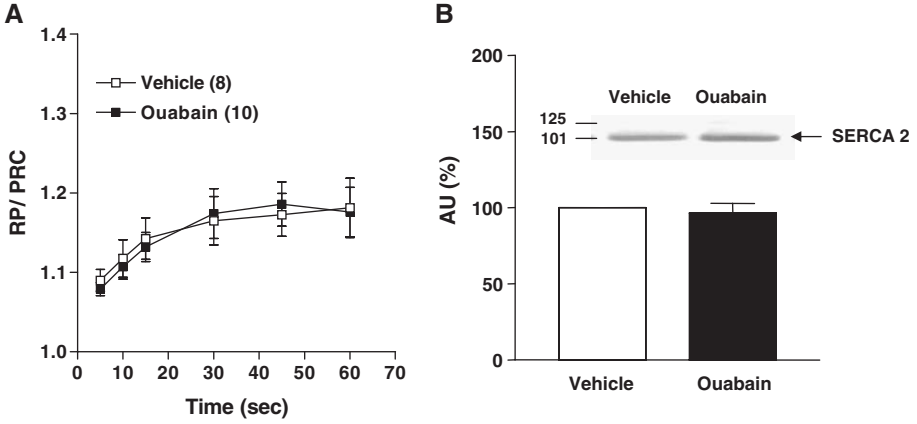

Fig. 2. (A) Changes of relative potentiation after post rest contractions (RP/PRC) and (B) densitometric analysis of the Western blot of SERCA-2 protein expression from left ventricle obtained from vehicle and ouabain-hypertensive rats. The inset in the (B) shows the representative Western blot for SERCA-2 protein expression of left ventricle obtained from vehicle and ouabain-hypertensive rats. Western blot results are expressed as a percentage of the signal of SERCA-2 protein of the corresponding left ventricle from vehicle-treated rats. Columns or symbols represent mean±SEM.

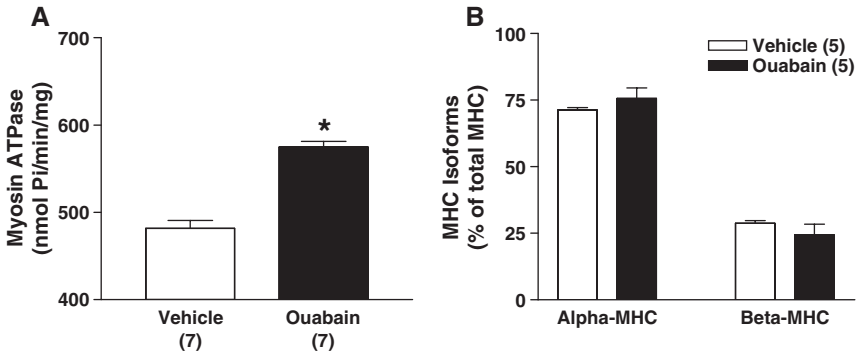

Fig. 3. Comparisons of myosin ATPase activity (A) and protein expression of alpha- and beta-myosin heavy chain (MHC) isoforms (B) of the left ventricle from vehicle and ouabain-hypertensive rats. Western blot results are expressed as a percentage of total myosin heavy chain (MHC) protein content present in the left ventricle from vehicle or ouabain-treated rats. Columns represent mean±SEM. $P<0.05$, *vs. vehicle.

treated muscle were observed (Fig. 2A). In addition, after 5 weeks of ouabain treatment no difference was observed for SERCA-2 protein expression when compared to the vehicle group (Fig. 2B).

The possibility of actions of chronic ouabain treatment on the contraction machinery was also studied by measuring the activity of myosin ATPase and the protein expression of myosin heavy chain isoforms. The activity of myosin ATPase was higher in the ouabain-hypertensive muscles (Fig. 3A). However, protein expression of both α and β isoforms of myosin heavy chain was similar in ouabain and vehicle groups (Fig. 3B).

As the main site of ouabain action is the catalytic subunits of Na^+, K^+-ATPase we also measured the protein expression of α_1 and α_2 isoforms of the Na^+, K^+-ATPase. Ouabain treatment increased both α_1 and α_2 protein expression of Na^+, K^+-ATPase in the left ventricle as compared to the vehicle group (Fig. 4).

Discussion

In this study we demonstrated for the first time that chronic treatment with ouabain for 5 weeks, which induces hypertension, is associated with an increase of the ventricular inotropic and lusitropic functions. This treatment did not produce cardiac hypertrophy nor altered the function of the sarcoplasmic reticulum but increased the activity of the myosin ATPase and the protein expression of the catalytic α_1 and α_2 isoforms of the Na^+, K^+-ATPase.

Similar to previous results (Huang et al., 1994; Manunta et al., 1994; Kimura et al., 2000; Rossoni et al., 2002; Di Filippo et al., 2003; Xavier et al., 2004; Briones et al., 2006) the present work reinforce the hypothesis that chronic treatment with ouabain in Wistar rats induces hypertension and did not change heart rate. As described in the Introduction, the mechanisms

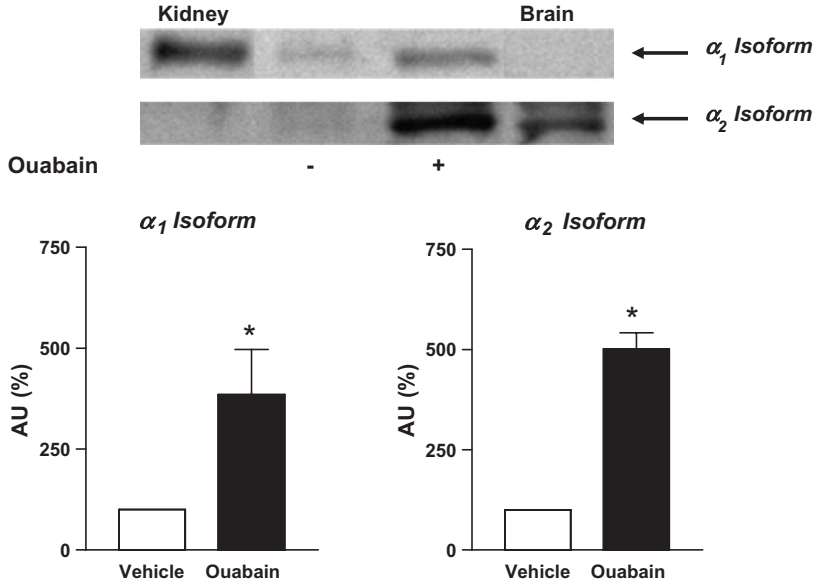

Fig. 4. Upper panel—representative Western blots for α_1 and α_2 isoforms protein expression in intact segments from left ventricle of Wistar rats, which received ouabain (+) or vehicle (−) subcutaneously for 5 weeks. The first and last line shows the corresponding positive control for each protein (kidney for α_1 and brain for α_2). Lower panel—densitometric analysis of the Western blot of α_1 and α_2 protein expression in intact segments from left ventricle of Wistar rats that received ouabain ($N=4$) or vehicle ($N=4$) for 5 weeks. Results are expressed as a percentage of the signal of the α_1 or α_2 isoforms of the corresponding left ventricle from vehicle-treated rats. $P<0.05$,*vs. vehicle.

involved on the genesis and/or maintenance of this model of hypertension are associated with changes in the central nervous system pathways (Huang et al., 1994; Huang and Leenen, 1999; Di Filippo et al., 2003) and in the arterial function and remodeling (Kimura et al., 2000; Rossoni et al., 2002; Di Filippo et al., 2003; Xavier et al., 2004; Briones et al., 2006). However, there are no reports describing changes in heart function in the ouabain-hypertensive rats.

In conscious rats, we found suggestions that the contractile activity was enhanced in ouabain-hypertensive animals because an increment of the LVSP and of the positive dP/dt_{max} was observed. In addition, this hypertensive treatment also increased lusitropic parameters as observed by an increment in negative dP/dt. Moreover, the isolated left ventricular papillary muscles from ouabain-hypertensive rats developed more force when compared to the vehicle group, which also could explain the increment of the positive dP/dt_{max} reflecting an increased inotropic state. This force increment is maintained even when the muscle is under inotropic interventions like increased extracellular Ca^{2+} concentration or changes in the rate of stimulation. This is an interesting finding that suggests that the positive inotropic action of ouabain can be superimposed to other positive inotropic actions without blunting them. Reinforcing these results, El-Armouche et al. (2004) showed in left ventricular papillary muscle from ouabain-treated rats for 4 days a sensitization for the positive inotropic effect of isoprenaline.

In the rat myocardium both positive inotropic and lusitropic effects are usually dependent on the activity of the sarcoplasmic reticulum. Older reports suggested that ouabain reduces calcium uptake (Lee and Choi, 1966; Carsten, 1967) and potentiates calcium release from the sarcoplasmic reticulum (Fugino et al., 1979; McGarry and Williams, 1993). More recently, Sagawa et al. (2002) reported that cardiac glycosides amplify sarcoplasmic reticulum calcium release by a luminal calcium sensitive mechanism. Acting together those mechanisms contribute to increase myoplasmic calcium concentration enhancing contraction. However, all these findings were obtained with acute ouabain administration.

We then investigated possible changes of the sarcoplasmic reticulum function, after chronic ouabain administration, using the relative potentiation, obtained after pauses of increasing duration performed in isolated left ventricular papillary muscles (Mill et al., 1992), and by measuring the SERCA-2 protein expression. Our findings did not reveal changes in the relative potentiation and these results were reinforced by the fact that SERCA-2 protein expression did not change after ouabain treatment. In agreement, Müller-Ehmsen et al. (2003) using cultured rat cardiomyocytes treated for 2 or 4 days with ouabain and El-Armouche et al. (2004) using the infusion of this compound for 4 days in Wistar rats also showed that these treatments did not change the transduction pathway involved in calcium handling as the expression of SERCA-2a, phospholamban, calsequestrin or ryanodine receptor. On the other hand, in spontaneously hypertensive rats (SHR) the larger force increment developed by papillary muscles, when compared to controls, is abolished after treatment with ryanodine (Mill et al., 1998). However, previous report showed that the enhanced contractility found in the SHR was not associated with any changes in the density of ryanodine receptors, Ca^{2+}-ATPase or phospholamban but to the larger average amplitude of Ca^{2+} sparks (Shorofsky et al., 1999).

Hypertension produces cardiac overload and it is usually associated with ventricular hypertrophy, and this hypertrophy is associated with changes in the contractile machinery as changes in myosin ATPase activity and in MHC isoforms (Mercadier et al., 1981; Swynghedauw, 1999). Knowing that changes in myosin ATPase activity can change the ventricular contractility we performed protocols to investigate this activity. Indeed, we observed for the first time an increment of myosin ATPase activity on the left ventricular tissue from ouabain-hypertensive rats. This fact could help to explain the enhanced contractile activity of papillary muscles in the control condition. We also investigated the isoforms of the myosin heavy chain but, different from the myosin ATPase activity, the protein expression of α and β isoforms did not change. The relationship among myosin ATPase activity with larger percentage of α-MHC and the isometric force development is a fact already established (Barany, 1967; Swynghedauw, 1986, 1999; Tanamura et al., 1993). However, in the ouabain-treated hearts the increments of myosin ATPase activity and of the isometric force development are not associated to changes on the MHC isoforms. Although existing this positive correlation between the myosin ATPase activity and the abundance of α-MHC there are other reports (Bartunek et al., 2000) showing that in L-NAME-treated rats cardiac inotropism can be increased without alteration of the isoform composition of the cardiac muscle, as reported for the ouabain treatment model.

Additionally, the present study also demonstrated that ouabain-induced hypertension occurs without cardiac hypertrophy, despite of increased left ventricular function and the elevated arterial blood pressure. This result is very interesting, although reports show that ouabain induces hypertrophy in isolated cardiac myocytes by partial inhibition of the Na^+, K^+-ATPase activity (Huang et al., 1997; Xie et al., 1999). Previously, we demonstrated that ouabain-induced hypertension is associated with regional changes in the activity of the ouabain-sensitive sodium pump and of the expression of the α_1 and α_2 Na^+, K^+-ATPase isoforms in the vascular smooth muscle (Rossoni et al., 2002). Kent et al. (2004) also showed that chronic treatment with ouabain for 14 days directly inhibits Na^+, K^+-ATPase activity in the hypothalamus and up-regulated the α_3 isoform expression while α_1 and α_2 expression remains unchanged. Our findings reinforce those results, because in the left ventricle from ouabain-hypertensive rats there is an increment in the α_1 and α_2 isoforms of Na^+, K^+-ATPase. Gao et al. (2002) showed that low concentration of digitalis compounds activate the cardiac Na^+, K^+-ATPase activity instead of inhibiting it. On the other hand, as described above, it is known that the myocyte's hypertrophy and transcriptional regulations of growth-related genes induced by in vitro ouabain treatment are partially dependent of the Na^+, K^+-ATPase inhibition (Huang et al., 1997; Xie et al., 1999). Supported by

these results, we can speculated that, in vivo, cardiac hypertrophy does not develop, as in cultured cardiac myocytes, probably because of the activation of α_1 and α_2 isoforms of the Na^+, K^+-ATPase by low doses of ouabain, as used in the present study.

In the rat heart approximately 75% of the alpha Na^+, K^+-ATPase protein isoform is α_1; however, α_2 and/or α_3 are consistently found in strategic sites (Blaustein et al., 1998). It is know that the main site for ouabain action is assumed to be the α-subunit of the Na^+, K^+-ATPase. The inhibition of the sodium pump results in Na^+ accumulation in the myoplasm which reduces the activity of the Na^+/Ca^{2+} exchange mechanism and ultimately increases cardiac and vascular smooth muscle contraction (Blaustein et al., 1998). If ouabain increases the protein expression of α_1 and α_2 isoforms of Na^+, K^+-ATPase, changes in the activity of the sodium pump could contribute to the increment in the inotropic effect occurring in the ouabain-hypertensive rats. In addition, recently it was demonstrated that acute infusion of ouabain acting on α_2 isoforms of Na^+, K^+-ATPase enhances the myocardial contractility in mice (Dostanic et al., 2005). Our results also agree with Xie et al. (1999) and Xie and Askari (2002), which suggest that it may be possible to dissociate the positive inotropic effects induced by ouabain from its cardiac hypertrophic effect.

Potential limitations of the study

In the present study we used fluid-filled manometric system as a method to perform the hemodynamic experiments in conscious animals. If we compared the present results with results performed using microtip pressure transducers we observed that the present values obtained with polyethylene catheter are lower when compared to those obtained with the microtip catheter (Capasso et al., 1992; Pacher et al., 2004; Samsamshariat et al., 2005). However, the results using the microtip catheter are commonly performed in anesthetized rats. As the anesthetic state changes the hemodynamic parameters in ouabain-hypertensive animals, we used the fluid-filled manometric system to perform the present experiments knowing about the resonance effect of this catheter and the dumping produced by this manometric system. However, as the fluid-filled manometric system was the same to perform all experiments and, in addition, the results obtained on the isolated left ventricle papillary muscle agree with the hemodynamic data we believe that the differences obtained between the groups in the present study are acceptable.

This is the first demonstration that hypertensive ouabain-treated rats present positive left ventricular inotropism and lusitropism. Our results suggest that the left ventricular inotropic effect observed in papillary muscle and in awake ouabain-hypertensive animals does not depend on changes of the sarcoplasmic reticulum function or hypertrophy development. Indeed, it is associated with an increased activity of the myosin ATPase and an increased protein expression of catalytic subunits of Na^+, K^+-ATPase. All these mechanisms are superimposed by an increment in the sympathetic activity (Huang et al., 1994; Huang and Leenen, 1999; Di Filippo et al., 2003) that potentiates the positive inotropic and lusitropic mechanisms in these hearts.

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