Original Paper

Myocardial Contractile Dysfunction Induced by Ovariectomy Requires AT₁ Receptor Activation in Female Rats

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Key Words

Ovariectomy • Losartan • SERCA2a • Phospholamban • Myocardial contractility • AT₁ Receptor • Superoxide anion production

Abstract

Background/aim: Estrogen deficiency induces myocardial contractile dysfunction and increases cardiovascular disease risk. However, the mechanism underlying this response is unclear. Our aim was to investigate whether AT₁ receptor blockade would prevent ovariectomy-induced myocardial contractile dysfunction. **Methods:** Female rats (8 weeks old, 280 g) that underwent bilateral ovariectomy were randomly assigned to receive daily treatment with losartan (OVX + LOS, 15 mg/kg, s.c., in 0.9 % NaCl), placebo (OVX), estrogen replacement (OVX + E2, 1 mg/kg, once a week, i.m.) and SHAM for 58 days. **Results:** Losartan and estrogen treatment 1) prevented ovariectomy-induced weight gain and slight hypertrophy, 2) restored the positive inotropic responses to Ca²⁺ and isoproterenol in the isolated papillary muscle in the OVX group, 3) prevented the reduction in SERCA2a levels and the increase in phospholamban (PLB) expression in the OVX group, 4) abolished the increase in superoxide anion that was increased in the OVX group, and 5) normalized the increase in p22^{phox} expression after ovariectomy. Estrogen treatment but not losartan restored the increase in serum angiotensin converting enzyme activity in the OVX group. **Conclusion:** This study demonstrated that myocardial contractile dysfunction induced by ovariectomy and expression of key Ca²⁺-handling proteins were prevented by losartan treatment and that AT₁ receptor activation is involved in this response.

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Introduction

Striking sex-based differences have been reported in the incidence of cardiovascular diseases and myocardial contractility [1-3]. Premenopausal women have a reduced risk of mortality from cardiovascular diseases, and postmenopausal women have a similar or even increased risk for cardiovascular diseases compared to men [2, 4]. Female sex hormones, especially estrogen, have been shown to be beneficial for cardiovascular function. Postmenopausal women on hormone replacement therapy (HRT) demonstrate better cardiac function than those without hormone supplements [5, 6]. Although estrogen replacement therapy has been demonstrated to reduce cardiovascular risks, controversies regarding the safety of HRT have drawn attention to new therapies for cardiovascular diseases [7, 8].

Estrogen deficiency plays a role in the contractile activity of the heart [9-11] by decreasing SERCA2a expression and its function, diminishing Ca^{2+} responsiveness of myofilament activation and increasing phospholamban expression [1, 7, 12]. We previously showed that estrogen deficiency induces myocardial contractile dysfunction in ovariectomized rats, but the mechanism underlying contractile protein regulation remains unclear [1, 7] because there are no estrogen response elements found on the gene encoding human SERCA2a [1, 13]. Moreover, there is no evidence that estrogen regulates transactivation directly through the hormone receptor bound to DNA [1, 14-16].

On the other hand, the protective effects of estrogen are mainly mediated through increased lipoprotein metabolism [17-20] and decreased serum and tissue angiotensin converting enzyme (ACE) expression and activity [20-22]. Proudler et al. [21] demonstrated that estrogen replacement therapy was able to decrease ACE activity and the risk of coronary artery disease in women. Studies in animals also showed that ACE activity appears to be affected by estrogen and the angiotensin II receptor [23]. According to Gallagher et al. [20], the reduction in ACE activity was not due to a direct interaction of estrogen with the enzyme; rather, it seems that estrogen regulates ACE mRNA synthesis at the tissue level. Thus, estrogen replacement therapy may contribute to enhanced cardiovascular protection by downregulating ACE, thereby reducing angiotensin II levels. In fact, angiotensin II is an important factor in cardiac remodeling and left ventricular dysfunction, and tissue angiotensin II is increased in failing hearts [24, 25]. Angiotensin II activates intracellular signaling that promotes adaptive responses to increased myocardial stresses, such as cell growth, hypertrophy, collagen synthesis and oxidative stress.

Additionally, angiotensin II induces diastolic dysfunction attributed to impaired SERCA2a activity [26-28] and increases NADPH oxidase expression and activity, thereby augmenting superoxide anion production [29], which decreases myofilament responsiveness to calcium and triggers negative inotropy [30].

Based on the evidence presented, our aim was to investigate whether AT1-receptor blockade and estrogen replacement would prevent ovariectomy-induced myocardial contractile dysfunction. We hypothesized that AT1 receptor activation is needed to induce myocardial contractile dysfunction through oxidative stress. Therefore, we analyzed the effects of these treatments on the following: 1) weight gain and hypertrophy induced by ovariectomy; 2) hemodynamic and myocardial contractility; 3) reactive oxygen species (ROS) production; 4) measurement of plasma ACE activity and 5) expression of key Ca^{2+} -handling proteins. Our findings provide evidence that angiotensin II receptor activation is the main cause of myocardial contractile dysfunction after ovariectomy in female rats.

Materials and Methods

Animal care

The care and use of the laboratory animals were in accordance with National Institutes of Health (NIH) guidelines and were approved by the local animal ethics committee. All rats had free access to tap water and food.

Experimental groups, surgical procedures and E2 replacement

Eight-week-old female Wistar rats were randomly divided into four groups. Rats underwent bilateral ovariectomy as described previously [31]. Briefly, rats were anesthetized, and a dorsal midline skin incision was made caudal to the posterior border of the ribs. The posterior abdominal muscle wall was bluntly dissected, the abdominal cavity was opened and the ovary was gently exteriorized and removed. The uterine horn was returned to the abdomen. The skin incision was closed with sterile nylon sutures, and the process was repeated on the other side. The rats were randomly assigned to receive treatment with losartan (OVX + LOS, 15 mg/kg in 0.9% NaCl, s.c., daily), placebo (OVX) or estrogen replacement (OVX + E2, 1 mg/kg, i.m., once a week), and the last group underwent a sham operation and served as normal controls (SHAM). All treatments lasted for 58 days.

Left ventricle papillary muscle contractility was studied 60 days postsurgery. At the time of sacrifice, the adequacy of the ovariectomy was grossly determined by the absence of ovarian tissue and marked uterine atrophy. We also determined the weight of the entire animal, as well as the weight of the left ventricle and the uterus.

Isometric tension and myocardial contractility

Rats received 500 units of heparin intraperitoneally (i.p.) and were anesthetized 10 minutes later with urethane 1.2 g/kg (Sigma). The hearts were rapidly removed and perfused through the aortic stump and the left ventricle papillary muscles were dissected. Muscle preparations were mounted for isometric tension recording and maintained in 20 mL Krebs-Henseleit solution (in mM: NaCl 118, KCl 4.7, CaCl₂ 1.25, KH₂PO₄ 1.2, MgSO₄ 1.2, NaHCO₃ 23 and glucose 11) at 30°C and pH = 7.4, which was continuously aerated with 95% O₂ and 5% CO₂. Resting tension was adjusted to produce maximal contractile force (Lmax). The twitch contraction rate was controlled by isolated rectangular pulses (10 to 15 V, 12 ms duration) through a pair of platinum electrodes. The standard stimulation rate was 0.5 Hz (steady-state). Isometric force development was measured with an isometric force transducer (TSD105A, Biopac) and normalized to muscle weight (g/g). Recording started after 60 minutes to permit the muscle to adapt to the new environmental conditions. Myocardial contractility was tested by the following protocol: first, we measured the inotropic response to changes in extracellular calcium (CaCl₂, Merck) concentration (0.62 to 3.75 mM); next, the positive inotropic responses, produced by increasing isoproterenol (Sigma) concentrations added to the bath (10⁻⁸ – 10⁻² M), were analyzed; finally, the papillary muscle was removed and weighed for force normalization (g/g).

Hemodynamic and ventricular compliance measurements

Animals were anesthetized with a mixture of the ketamine/xylazine (90 mg/kg and 10 mg/kg, respectively). The right carotid artery was isolated, and a polyethylene catheter (PE-50) was inserted and connected to a data acquisition system to measure the hemodynamic parameters. Following an adaptation period of 30 min, arterial pressure acquisition started. The catheter was advanced into the left ventricle, and systolic pressures (LVSP), end-diastolic pressures (LVEDP), dP/dt +, dP/dt - and heart rate (HR) were continuously monitored for 15 min. To define the passive pressure-volume characteristics of the left ventricle, the heart was arrested in diastole with potassium chloride (1 M) and a double lumen catheter (PE 50 inside PE 200) was inserted 6 mm into the left ventricle via the aorta. The right ventricular free wall was incised to avoid fluid accumulation and variable compressive force on the interventricular septum. The atrioventricular groove was tied, and the ventricle was manually compressed to expel blood and create a negative pressure of -5 mm Hg, which was taken as zero volume. Physiological saline was infused at 0.68 mL/min via one lumen, while the intraventricular pressure was continually recorded through the other lumen over the pressure range of -5 to 30 mmHg. At least three reproducible pressure-volume curves were obtained within 10 minutes of cardiac arrest and well before the onset of rigor mortis. Ventricular volumes at pressures of 0, 2.5, 5, 10, 15, 20, and 30 mmHg were determined for the pressure-volume curve.

The obtained curves were segmented and then separately analyzed. Pressure follows a linear pattern from 0 to 5 mmHg during volume infusion, and the slope is indicative of left ventricular dilatation. For the 5 to 30 mmHg segment, the curve was adjusted to a monoexponential model. Thus, to determine the stiffness constant during the 5-30 mmHg interval, a logarithmic transformation was performed on the pressure scale to create a linear fit.

Western blot analysis

Proteins from homogenized hearts (50 µg for PLB and 80 µg for the other proteins) were separated by 7.5%, 10% or 15% SDS-PAGE. Proteins were transferred to nitrocellulose membranes, which were incubated with mouse monoclonal antibodies for SERCA2a (1:1000, Affinity BioReagents, CO, USA), PLB (0.5 µg/ml, Affinity BioReagents, CO, USA), phosphorylated PLB at serine 16 (1:5000, Badrilla, UK), phosphorylated PLB at threonine 17 (1:5000, Badrilla, UK), AT₁ receptor (1:1000, Santa Cruz Biotechnology) and AT₂ receptor (1:750, Santa Cruz Biotechnology). After washing, the membranes were incubated with anti-mouse (1:5000, Stressgen, Victoria, Canada) or anti-rabbit (1:7000, Stressgen, Victoria, Canada) immunoglobulin antibodies

conjugated to horseradish peroxidase. After thorough washing, immunocomplexes were detected using an enhanced horseradish peroxidase/luminal chemiluminescence system (ECL Plus, Amersham International, Little Chalfont, UK) and film (Hyperfilm ECL International). Signals on the immunoblot were quantified with the NIH Image V1.56 computer program. Each membrane was reprobed to determine GAPDH expression using a mouse monoclonal antibody (1:5000, Abcam Cambridge, MA, USA).

In situ detection of papillary O_2^- production

The oxidative fluorescent dye dihydroethidium (DHE) was used to evaluate O_2^- production in situ, as previously described [32]. Dihydroethidium freely permeates cells and is oxidized in the presence of O_2^- to ethidium bromide, which is trapped by intercalation with DNA. Ethidium bromide is excited at 546 nm and emits at 610 nm. Frozen tissue segments were cut into 10- μ m-thick sections and placed on glass slides. Serial sections were equilibrated under identical conditions for 30 min at 37°C in Krebs-HEPES buffer (in mM: 130 NaCl, 5.6 KCl, 2 CaCl₂, 0.24 MgCl₂, 8.3 HEPES, and 11 glucose, pH = 7.4). Fresh buffer containing DHE (2 μ M) was applied topically to each tissue section, covered with a cover slip, incubated for 30 min in a light-protected humidified chamber at 37°C, and then viewed with a fluorescent microscope (Nikon, 200x magnification) using the same imaging settings for all sections. Fluorescence was detected with a 568-nm long-pass filter. For quantification, 8 tissue sections per animal were sampled and averaged for each experimental condition. The mean fluorescence densities in the target region were calculated.

Measurement of MDA production

Plasmatic malondialdehyde (MDA) levels were measured with a modified thiobarbituric acid (TBA) assay [32]. Plasma was mixed with 20 % trichloroacetic acid in 0.6 M HCl (1:1 vol/vol), and tubes were incubated on ice for 20 min to precipitate plasma components that present possible interferences. Samples were centrifuged at 1,500 $\times g$ for 15 min before adding TBA (120 mM in 260 mM Tris, pH = 7) to the supernatant in a proportion of 1:5 (vol/vol), and the mixture was boiled at 97°C for 30 min. Spectrophotometric measurements at 535 nm were performed at 20°C.

Measurement of plasma ACE activity

Angiotensin converting enzyme activity was measured with a fluorometric method. Briefly, triplicate plasma samples (3 μ L) were incubated for 15 minutes at 37°C with 40 μ L of assay buffer containing the ACE substrate 5 mM Hip-His-Leu (Sigma). The reaction was stopped by adding 190 μ L of 0.35 N HCl. The resulting product (His-Leu) was fluorometrically measured following a 10 min incubation with 17 μ L of 2 % o-phthal-dialdehyde in methanol. Fluorescence measurements were carried out at 37°C in a plate reader (Synergy 2, Biotek) with 350 nm excitation and 520 nm emission filters. The fluorescence plate reader was controlled by the Gen 5 Software. Black 96-well polystyrene microplates (Corning Incorporated, NY, USA) were used. A calibration curve with His-Leu (Sigma) was included in each plate.

Immunohistochemical Analysis

Immunohistochemical localization of p22^{phox} was determined in 10- μ m sections of frozen papillary muscle using an anti-p22^{phox} (1:50, Santa Cruz Biotechnology) antibody. Briefly, frozen sections were fixed in cold acetone for 5 minutes, followed by pretreatment with 0.3 % hydrogen peroxide for 20 minutes to inhibit endogenous peroxidase activity. Subsequently, sections were blocked with 5% horse serum for 60 min and incubated with the primary antibody overnight at 4°C. After rinsing with PBS, the sections were incubated for 30 min with a biotinylated secondary antibody, followed by incubation with avidin-biotinylated horseradish peroxidase complex (Vectastain ABC, Vector). Serial sections treated with nonimmune IgG did not show any staining. Peroxidase staining was detected using the DAB detection system (Vector Laboratories).

Statistical analysis

All values are expressed as the means $\pm$ SEM. Differences among groups were analyzed using one- or two-way ANOVA (repeated measures) followed by the Bonferroni post hoc test for multiple comparisons. A p-value < 0.05 was considered significant. For protein expression, the data are expressed as the ratio between the protein of interest and GAPDH.

Drugs and chemicals

All chemicals, unless otherwise specified, were purchased from Sigma Chemical (St. Louis, MO) or Merck (Germany).

	SHAM	OVX	OVX + LOS	OVX + E2
BW (g)	256.8 ± 4.80	310.4 ± 8.80*	277.4 ± 3.40#	251.4 ± 9.50#
LV/BW (mg/g)	2.0 ± 0.03	1.9 ± 0.04*	1.8 ± 0.03*#	1.93 ± 0.05
Uterus/BW	2.5 ± 0.20	0.8 ± 0.05*	0.6 ± 0.03*	2.5 ± 0.19#
Tibia (mm)	38.0 ± 0.40	38.5 ± 0.30	38.2 ± 0.20	37.3 ± 0.10
LV/Tibia (mg/mm)	14.2 ± 0.20	15.4 ± 0.40*	13.3 ± 0.20#	13.05 ± 0.16#
Papillary muscle (mg)	5.2 ± 0.35	5.9 ± 0.50	6.1 ± 0.40	5.9 ± 0.50

Table 1. Ponderal data from SHAM, Ovariectomy (OVX), Ovariectomy plus losartan (OVX + LOS) and estrogen replacement (OVX+E2) groups. BW = body weight, LV/BW = left ventricle/body weight ratio, Uterus/BW = Uterus/body weight ratio, LV/Tibia = left ventricle/tibia ratio. * Significantly different from SHAM and # from OVX ($p < 0.05$) using one-way ANOVA and post hoc Bonferroni test

Results

Body, Heart and Uterus Weight

The body weights of ovariectomized rats were significantly greater than those of SHAM rats. Estrogen replacement and losartan treatment prevented weight gain after ovariectomy (Table 1). Estrogen levels were restored to the same levels as the control in the E2 replacement group (SHAM: 127 ± 11.5 , OVX: $50.4 \pm 11.4^*$ and OVX + E2: 142.2 ± 31 pmol/L, $*p < 0.05$). Ovariectomy and losartan treatment decreased left ventricle weight to body weight ratio. When we evaluated the left ventricle weight to tibia length ratio, rats in the OVX group exhibited a significantly higher ratio compared to SHAM, and losartan treatment or E2 replacement restored this ratio to the same level as the SHAM group (Table 1).

The deficiency of estrogen in OVX and OVX + LOS rats induced a significant decrease in uterine weight compared to SHAM controls. Restoration of uterine mass was observed in the E2 treated group. The tibia length or papillary muscle weight did not differ among groups (Table 1).

Isometric contractile response to calcium

As expected, increases in extracellular calcium concentration resulted in a positive inotropic response (Fig. 1). We found a difference in the inotropic response to calcium between the OVX and SHAM groups for all concentrations studied (Fig. 1A); the OVX group response is approximately 50 % less compared to the SHAM rats. The OVX group also presented a reduction in EC50 compared to SHAM group (SHAM: 342 ± 25 , OVX: $194 \pm 18^*$ and OVX + LOS: $317 \pm 30^{\#}$ and OVX + E2: $314 \pm 39^{\#}$, (g/g) $^{**}p < 0.05$). Interestingly, losartan treatment or estrogen replacement restored the estrogen deprivation-induced reduction in the inotropic response to extracellular Ca^{2+} (Fig. 1A, B).

Isometric contractile response to β -adrenergic receptor stimulation

Dose-response curves were measured for isoproterenol, a nonspecific β -adrenergic agonist (Fig. 1C and D). β -adrenergic receptor stimulation in the heart increases contractility and accelerates relaxation by activating the adenylyl cyclase/cAMP/protein kinase pathway [33]. As expected, isoproterenol promoted a positive inotropic effect in all groups studied. However, this response was reduced in the OVX group compared with SHAM controls by approximately 50 % (Fig. 1C, D).

The OVX group also presented a reduction in EC50 compared to SHAM group (SHAM: 312 ± 17 , OVX: $159 \pm 11^*$ and OVX + LOS: $293 \pm 28^{\#}$ and OVX + E2: $287 \pm 23^{\#}$, (g/g) $^{**}p < 0.05$). Both losartan treatment and E2 replacement restored the inotropic response to isoproterenol (Fig. 1C, D).

Fig. 1. Effects of increasing extracellular calcium concentration from 0.62 to 3.75 mM (A and B) and of treatment with increasing concentrations (10^{-8} to 10^{-2} M) of the β -adrenergic agonist isoproterenol (C and D) on force development in isolated left ventricle papillary muscles from SHAM, ovariectomized (OVX), ovariectomized plus losartan (OVX + LOS) and estrogen replacement (OVX + E2) rats 60 days postsurgery. Rmax and pD2 to isoproterenol and calcium from developed force. Force (g/g) values are expressed as the means $\pm$ S.E.M. * Significantly different from SHAM and # from OVX ($p < 0.05$) using two-way ANOVA, repeated measures and post hoc Bonferroni test.

Fig. 2. Angiotensin converting enzyme (ACE) activity (nmol His-Leu/ ml^{-1}) in plasma (top) and plasmatic malondialdehyde (MDA) levels (bottom) of SHAM, ovariectomized (OVX), ovariectomized plus losartan (OVX + LOS) and estrogen replacement (OVX + E2) rats at 60 days postsurgery. The values are expressed as the means $\pm$ S.E.M. * Significantly different from SHAM and # from OVX ($P < 0.05$) using one-way ANOVA and post hoc Bonferroni test.

Plasmatic ACE activity, Papillary Superoxide Anion Production and plasmatic MDA levels

After 8 weeks, plasma ACE activity was 2.8-fold greater in the OVX and losartan treated groups compared to SHAM control rats. E2 replacement restored the increase induced by estrogen deprivation to normal levels (Fig. 2).

Basal superoxide anion production was 4-fold higher in papillary segments from OVX rats than in SHAM rats. Both losartan and E2 treatments prevented the rise in superoxide production in papillary segments from OVX + LOS rats. Moreover, E2 replacement restored this increase induced by ovariectomy to normal levels (Fig. 3).

Fig. 3. Superoxide anion production in papillary muscle of SHAM, ovariectomized (OVX), ovariectomized plus losartan (OVX + LOS) and estrogen replacement (OVX + E2) rats at 58 days postsurgery. The values are expressed as the means $\pm$ S.E.M. * Significantly different from SHAM and # from OVX ($p < 0.05$) using one-way ANOVA and post hoc Bonferroni test.

Plasmatic MDA levels did not differ among the SHAM, OVX and OVX + E2 replacement groups. However, the losartan group exhibited a lower MDA level compared to the SHAM group (Fig. 2). These results suggest that activated AT_1 receptors are involved in the increased oxidative stress observed in OVX rats.

Western blot analysis

Alterations in cardiac mechanical properties and intracellular calcium homeostasis are dependent on SERCA2a and PLB. In this study, we examined the role of these proteins in the altered myocardial contractility seen in rats 60 days after ovariectomy. As shown in Fig. 4, SERCA2a protein expression is approximately 50% lower in the OVX group (Fig. 4A), and PLB protein expression is increased (Fig. 4B). E2 administration or losartan treatment effectively prevented these changes (Fig. 4A, B). Fig. 4 also demonstrates that the PLB levels in the OVX group were 1.7-fold higher than the SHAM group. The ratio of SERCA2a to PLB protein levels was significantly reduced (approximately 72 %) in ovarian sex hormone-deficient hearts (Fig. 4C). To examine the modulating effect of PLB on the responsiveness of SERCA2a to Ca^{2+} in ovariectomized hearts, the levels of PLB phosphorylated at either the Ser¹⁶ or Thr¹⁷ was analyzed. We did not find any difference among the groups in the content of the phosphorylated form of PLB at Ser¹⁶ (Fig. 4E), but we did find an increase in the content of the phosphorylated form at Thr¹⁷ in the OVX group compared to SHAM group. Losartan or estrogen treatment restored these changes (Fig. 4F).

Furthermore, we analyzed the expression of the AT_1 and AT_2 receptors and found an increase in AT_1 receptor protein expression in the losartan-treated group but no change among the other groups (Fig. 4D). AT_2 protein expression did not differ among the groups (data not shown).

Fig. 4. Densitometric analysis of Western blots for (A) SR Ca²⁺-ATPase (SERCA2a), (B) phospholamban (PLB), (C) SERCA2a/PLB ratio, (D) AT₁ receptor, (E) phosphorylated phospholamban at Ser¹⁶ (PLB^{Ser16}) and phosphorylated phospholamban at Thr¹⁷ (PLB^{Thr17}) in hearts from SHAM, OVX, OVX + LOS and OVX + E2 rats. * *p* < 0.05 by ANOVA vs. SHAM rats. # *p* < 0.05 by ANOVA vs. OVX rats. N = 5-13. Representative blots are shown.

Immunohistochemical Analysis

Immunohistochemistry for p22^{phox} was performed in serial sections to define the spatial distribution of p22^{phox} protein expression in papillary muscle. As illustrated in Fig. 5, immunoreactivity for p22^{phox} was readily detected in OVX hearts. Losartan or estrogen treatment diminished these ovariectomy-induced increases in protein expression in papillary muscle.

Pressure-Volume Data

Passive pressure-volume curve could be analyzed in 2 segments: the first linear segment representing the dilatation constant, and an exponential segment indicating left ventricular stiffness. Ventricular volumes or ventricular stiffness measured in the potassium-arrested heart did not differ among groups of rats at transmural pressures from 2.5 to 30 mm Hg.

Left ventricular dilatation or ventricular stiffness was similar among all groups, showing that either OVX or losartan treatment did not change these parameters (Fig. 6).

Discussion

Estrogen plays an important role in cardiovascular function. Our study demonstrates that immediate AT₁ receptor blockade prevents cardiac contractile dysfunction and oxidative stress after ovariectomy. This response was associated with changes in expression levels of 2 key proteins involved in calcium homeostasis, SERCA2a and PLB. Losartan normalized ovariectomy-induced contractile dysfunction, restored the SERCA2a and PLB levels and diminished reactive oxygen species formation. Furthermore, AT₁ receptor blockade attenuated the increase in p22^{phox} expression but did not affect the increased ACE activity in the OVX group.

The progression and mortality of cardiovascular diseases differ in premenopausal women compared to men of the same age [2, 4, 23], and the prognosis for women is

Fig. 5. Immunohistochemistry of p22^{phox} in papillary muscle. Photomicrographs of short-axis cross-sections of papillary muscle from representative animals in the SHAM, OVX, OVX + LOS and OVX + E2 groups that were immunostained for p22^{phox} with DAB (brown). Regions of interest are shown at higher magnification (200x). Strong p22^{phox} immunoreactivity was noted in OVX group. In losartan- and E2-treated rats, p22^{phox} immunoreactivity was dramatically decreased in the papillary muscle.

Fig. 6. Left ventricular in situ pressure-volume curve normalized for body weight. The chamber dilation, obtained in the first segment (0 to 5 mmHg) of the curve and chamber stiffness was obtained in the second segment (5 to 30 mmHg) of the curve. N= 6-10. The data are expressed as the means $\pm$ S.E.M. No difference was found using two-way ANOVA, repeated measures and post hoc Bonferroni test.

significantly better than for men after the onset of cardiovascular disease [34], showing that premenopausal women are essentially protected from cardiovascular disease [35].

Estrogen deficiency has been related to myocardial contractile dysfunction. In agreement with other studies, our results suggest that estrogen deprivation-induced myocardial contractile dysfunction is linked to alterations in SERCA2a and PLB expression [1, 7, 8]. Phospholamban regulates SERCA2a activity by modulating calcium uptake into the sarcoplasmic reticulum and muscle relaxation [7, 36-38]. *In vivo* studies using transgenic mice that overexpress cardiac specific PLB suggested that the “functional stoichiometry” of PLB/SERCA2 is less than 1:1 in native cardiac sarcoplasmic reticulum membranes [39]. Here, we show that this ratio is three-fold higher in the OVX group compared to the sham group. According to Kadambi et al., [39] two-fold higher PLB protein levels in transgenic mice compared to WT resulted in decreased Ca²⁺-ATPase affinity for Ca²⁺, which was associated with decreased contractility and Ca²⁺ transport in cardiomyocytes.

Furthermore, losartan and estrogen treatment prevented the decrease in SERCA2a and the increase in PLB protein expression, providing evidence that AT₁ receptor activation may be responsible for estrogen deprivation-induced alterations.

Studies have suggested that estrogen regulates SERCA2a and PLB expression [1, 13]. The mechanism is unclear, but our results suggest that AT₁ receptor is involved in this response because losartan prevented the increase in PLB and the decrease in SERCA2a expression.

In fact, estrogen down-regulates most of the components of the renin angiotensin system, including ACE and AT₁ receptor [20, 28, 40]; therefore, estrogen deficiency leads to upregulation of AT₁ and its affinity for angiotensin II [41].

Domenighetti et al. [26] showed that overexpression of angiotensin II in the heart induces cardiomyocyte remodeling such as hypertrophy, decreases SERCA2a expression and leads to contractile dysfunction. We found that slight hypertrophy after ovariectomy was prevented by either losartan or estrogen, and it seems that cardiac hypertrophy is more dependent on hemodynamic changes than on local angiotensin levels [25]. As blood pressure or ventricular stiffness did not change, these animals would not be expected to

have hypertrophic hearts. Angiotensin II most likely negatively affects excitation-contraction coupling in the myocardium by increasing superoxide production in the heart [30]. Estrogen may play a more important role in mediating oxidative stress in males than in females [42]. Prolonged exposure to angiotensin II activates the Ca^{2+} /calmodulin-dependent protein kinase (CaMK) II by increasing reactive oxygen species [43] which in turn phosphorylates phospholamban at Thr17, increasing calcium reuptake by SERCA2a and leading to cell death [44]. These findings can explain the reason time to peak tension or time of relaxation were not prolonged in the ovariectomized group as compared to SHAM.

The main source of reactive oxygen species activated by angiotensin II is the enzyme NADPH oxidase, which is associated with a smaller protein called p22^{phox} that stabilizes the NOX complex [45]. Dantas et al. [46] reported a gender difference in NADPH oxidase activation in the vascular wall of hypertensive animals, showing that female animals have reduced levels of p22^{phox} and superoxide anion production. Reactive oxygen species are capable of influencing calcium homeostasis and cellular signaling via posttranslational modifications, such as S-glutathionylation, which inhibits calcium-induced calcium release in rat cardiac myocytes [47]. In fact, reactive oxygen species can modulate calcium handling proteins [48], such as ryanodine receptors, SERCA2a and L-type calcium channels, thereby contributing to slowed calcium transients [49-53]. In the present study, we observed increased levels of oxidative stress and PLBThr¹⁷ phosphorylation, as reported by others [30, 42, 46]. Oxidative stress may have contributed to the myocardial contractile dysfunction observed in OVX rats.

It has been suggested that estrogen modulates NADPH oxidase activity and expression; the p22^{phox} subunit is activated when cardiac hypertrophy progresses to heart failure, as well as in other diseases, including atherosclerosis and myocardial infarction [46, 54]. Our results show that the OVX group had a higher content of p22^{phox} compared to control animals, and losartan or estrogen were able to prevent this estrogen deficiency-induced increase.

In summary, our data indicate that treatment with losartan and estrogen replacement prevented the weight gain, slight hypertrophy and myocardial contractile dysfunction induced by ovariectomy. Moreover, this myocardial contractile dysfunction appeared to be dependent on superoxide anion production, which has detrimental effects on SERCA2a and PLB levels. Taken together, our findings provide evidence that angiotensin II receptor activation is the main cause of myocardial contractile dysfunction in ovariectomized female rats.

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