Ovariectomy augments pressure overload-induced hypertrophy associated with changes in Akt and nitric oxide synthase signaling pathways in female rats

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Bhuiyan MS, Shioda N, Fukunaga K. Ovariectomy augments pressure overload-induced hypertrophy associated with changes in Akt and nitric oxide synthase signaling pathways in female rats. Am J Physiol Endocrinol Metab 293: E1606–E1614, 2007. First published September 18, 2007; doi:10.1152/ajpendo.00246.2007.—To elucidate the molecular mechanism underlying estrogen-mediated cardioprotection in left ventricular (LV) hypertrophy and remodeling, we analyzed myocardial hypertrophy as well as cardiac function and hypertrophy-related protein expression in ovariectomized, aortic-banded rats. Wistar rats subjected to bilateral ovariectomy (OVX) were further treated with abdominal aortic stenosis. Effects on LV morphology and function were assessed using echocardiography, and expression of protein levels was determined by Western blot analysis. The heart-to-body weight ratio was most significantly increased in the OVX-pressure overload (PO) group compared with the OVX group and in the PO group compared with sham. The LV weight-to-body weight ratio was also significantly increased in the OVX-PO group compared with the OVX group and in the PO group compared with sham. The most significant increases in LV end diastolic pressure, LV developed pressure, and ±dp/dt max were observed in the OVX-PO group compared with the OVX group and represent compensatory phenotypes against hypertrophy. Both endothelial nitric oxide (eNOS) synthase expression and activity was markedly reduced in the OVX-PO group, and protein kinase B (Akt) activity was largely attenuated. Marked breakdown of dystrophin was also seen in hearts of OVX-PO groups. Finally, significantly increased mortality was observed in the OVX-PO group following chronic isoprotrenol administration. Our results demonstrate that rats subjected to ovariectomy are unable to compensate for hypertrophy, showed deteriorated heart function, and demonstrated increased mortality. Simultaneous impairment of eNOS and Akt activities and reduced dystrophin by ovariectomy likely contribute to cardiac degeneration during PO-induced hypertrophy in ovariectomized rats.

estrogen; myocardial hypertrophy; nitric oxide synthase

EPIEMIOLOGICAL STUDIES SHOW that the incidence of cardiovascular disease is higher in men than in premenopausal women but increases in postmenopausal women (10). Sex differences in left ventricular (LV) hypertrophy and remodeling have also been observed in aging and pressure-overloaded human hearts (4). It is suggested that, because of reduced estrogen levels after menopause, women lose an important cardiovascular protective mechanism and are at greater risk of developing hypertension (1). Moreover, estrogen has multiple protective effects on the cardiovascular system (27). However, the role of estrogen in development of cardiac hypertrophy is poorly understood. Most animal models of heart failure indicate that females resist cardiac contractile dysfunction (8, 11, 34). The relationship of sex and hypertrophy-induced heart failure is complex and depends on the model/etiology of hypertrophy, age, and stage of heart failure. Sex studies of the cardiovascular system have focused on estrogen’s vasoprotective effects (27); however, it is now clear that estrogen has direct effects on the myocardium independent of its vasoprotective effect. Specifically, Van Eickels et al. (45) found that estradiol attenuates development of pressure overload-induced hypertrophy in ovariectomized mice.

The biological function of estrogens is mediated by two different estrogen receptor (ER) subtypes, estrogen receptor α (ERα) and estrogen receptor β (ERβ), which were functionally expressed in cardiac myocytes and cardiac fibroblasts (12). Previous studies have shown that ovariectomy causes adverse LV remodeling by downregulation of ERα and ERβ in rats. Estrogen replacement therapy in these ovariectomized rats protects from adverse LV remodeling by upregulation of both the ERα and ERβ, suggesting estrogen-mediated cardiovascular protective effects via mechanisms involving ERs (48). Moreover, it has been reported previously that estrogen may inhibit the development of LV hypertrophy in sinoaortic-deververated rats (2) and that, in ovariectomized mice subjected to pressure overload hypertrophy, estrogen replacement attenuated the hypertrophic response (45). In addition to binding to the ligand-activated transcription factors (ERα and ERβ), estrogen can also bind to plasma membrane heptahelical G protein coupled receptors (GAP30) and activate additional signaling pathways such as activation of the phosphatidylinositol 3-kinase (PI 3-kinase), protein kinase B (Akt), and endothelial nitric oxide synthase (eNOS) pathways (14, 30). Of potential importance for cardioprotection is the synergy between the ER and GPR30 pathways. For example, ER pathways have been shown to lead to upregulation of eNOS, whereas GPR30 signaling results in activation of Akt and increased phosphorylation and activity of eNOS (30).

Nitric oxide (NO) production stimulated by estrogen is known to play an important role in the pathogenesis of sex-based differences in cardiac disease (31). Indeed, estrogen regulates expression of both eNOS and inducible nitric oxide synthase (iNOS) in neonatal and adult cardiac myocytes (32). Cardiac NO synthesis improves cardiac muscle function (26). Moreover, estrogen treatment increases eNOS activity in guinea pig heart (47); however, regulation of other nitric oxide synthase (NOS) isoforms in the myocardium by estrogen is poorly understood.

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Both PI 3-kinase/Akt (22, 40) and extracellular signal-regulated kinase (ERK) pathways (32) are downstream targets of nonnuclear estrogen signaling. For example, estrogen activates eNOS activity through ERK and Akt pathways, leading to enhanced NO release (40). Moreover, estrogen-mediated eNOS activation resulting from Akt activation has been demonstrated in cultured human endothelial cells (20) and in intact elastic and muscular arteries in vivo (18, 20). Similarly, induction of eNOS and iNOS by estrogen in cardiac myocytes is blocked by the mitogen/extracellular-regulated kinase inhibitor PD-98059 (32, 33). Induction of NOSs inhibits caspase activation and blocks development of congestive heart failure (29). Taken together, we hypothesized that NO generated from NOSs may play a pivotal role in sex-mediated cardioprotection.

Here, we asked whether ovariectomized (OVX) female rats show differences from sham-operated animals in compensatory phenotypes against pressure overload-induced cardiac hypertrophy. To induce pressure overload-induced hypertrophy, we used abdominal aortic constriction because that model causes reproducible hypertrophy resembling that induced by aortic stenosis and essential hypertension (23, 36). We also examined the effects of ovariectomy on compensatory phenotypes in cardiac contractility and cell survival signaling. We found that cardiac decompensation in survival signaling and impairment of contraction-related proteins contributed to increased mortality when animals were exposed to isoproterenol stimulation.

**MATERIALS AND METHODS**

**Materials.** Reagents and antibodies were obtained from the following sources: L-[2,3,4-3H]arginine (PerkinElmer Life Sciences, Boston, MA); anti-eNOS antibody (Sigma, St. Louis, MO); anti-neuronal nitric oxide synthase (nNOS) and anti-iNOS antibody (BD Biosciences, San Jose, CA); anti-phospho-Akt antibody (Ser473) and total-Akt antibody (Cell Signaling Technology, Beverly, MA); anti-phospho-Akt antibody (Thr308) (Upstate Biotechnology, Lake Placid, NY); anti-p53 mitogen-activated protein kinase (MAPK) and anti-phospho-p38-MAPK antibody (Thr180/Tyr182) (New England Bio-\textsc{\text}tsins, Osaka, Japan).

**Animals.** Female Wistar rats (6 wk old) weighing 150–200 g were obtained from Japan SLC (Hamamatsu, Japan). Rats were housed under climate-controlled conditions with a 12:12-h light-dark cycle and were provided with standard food and water ad libitum. An acclimation period of at least 1 wk was provided before initiating the experimental protocol. All procedures for handling animals were approved by the Animal Experimentation Committee of Tohoku University Graduate School of Pharmaceutical Sciences.

**Experimental surgical procedure.** Female rats were randomly separated into the following four treatment groups: 1) control (n = 7), 2) ovariectomy (OVX; n = 7), 3) pressure overload (PO; n = 8), and 4) ovariectomy plus pressure overload (OVX-PO; n = 8). Bilateral ovariectomy was produced in rats anesthetized with pentobarbital sodium (50 mg/kg ip; Tokyo Kasei Kogyo, Tokyo, Japan; see Ref. 24). A sham operation was performed by exposing the ovaries without isolation.

After the sham operation or ovariectomy (2 wk), PO cardiac hypertrophy was initiated in both groups by abdominal aortic banding as described by Jouannot and Hatt (23), with minor modifications. Briefly, rats were anesthetized, the abdominal aorta was exposed under sterile conditions through a midline abdominal incision, and a blunted 25-gauge needle (outside diameter, 0.5 mm) was placed between the right and left renal arteries. A ligature (6-0 silk) was snugly tied around both the renal artery and the needle. The needle was then removed, leaving the internal diameter of the aorta approximately equal to that of the needle. Sham-operated animals had an untied ligature placed in the same location. After surgery, animals were housed under controlled environmental conditions with food (Purina FormulaLab Chow 5008) and water ad libitum.

**Serum estrogen concentration measurements.** Serum estrogen levels in the sham, OVX, PO, and OVX-PO groups were analyzed by enzyme immunoassay according to the manufacturer’s instructions (Estradiol EIA kit; Cayman Chemical, Ann Arbor, MI).

**Hemodynamic measurements.** Hemodynamic measurements were done as described by Feng et al. (13). Briefly, rats were anesthetized with pentobarbital sodium (50 mg/kg ip). The right carotid artery was cannulated with a polyethylene catheter (PE-50, length 25 cm) filled with degassed saline containing heparin (300 IU/ml). Hemodynamic variables were measured with a pressure transducer (Nihon Kohden) connected to a polygraph (Nihon Kohden) and recorded using a thermal recorder (Nihon Kohden). Meticulous care was taken to ensure that the system remained free of air bubbles during the experimental period. After arterial blood pressure (AP-601G; Nihon Kohden) and heart rate (AT-601G; Nihon Kohden), measurements were obtained through the polygraph, the catheter was advanced to the LV cavity. We then measured LV systolic and end-diastolic pressures (AP-601G; Nihon Kohden), the maximal rate of pressure development (+dp/dt), and the rate of relaxation (−dp/dt) of LV by cardiotachometer (EQ-601G; Nihon Kohden) using the polygraph.

**Western blot analysis.** After aortic banding (4 wk), rats were anesthetized, and hearts were excised and quickly perfused with PBS to wash out blood from coronary vessels. Heart tissue was sliced at 2-mm thickness using a slicer (RBS-2; Zivic-Miller Laboratories, Zelienople, PA). LV tissue samples were then rapidly frozen in liquid nitrogen and stored at −80°C before use. For assays, each frozen sample was homogenized by methods described previously (43). An equal amount of protein for each sample (25 μg of total protein) was separated on 7.5–15% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). After being blocked with 5% low-fat milk in Tris-buffered saline plus Tween 20, membranes were incubated with specific primary antibodies overnight at 4°C. This was followed by a donkey anti-rabbit IgG coupled to horseradish peroxidase, and the blots were developed using the enhanced chemiluminescence immunoblotting detection system (Amersham Biosciences) and visualized on X-ray film (Fuji Film). Autoradiographic films were scanned by densitometry (Lasergraphics, Irvine, CA) and quantitated using Imagegauge V3.41 (Fuji Film). The relative amounts of proteins were expressed as percent increase over sham.

**Determination of eNOS activity.** To measure activity of eNOS, which is localized in the membrane/microsomal fractions, the LV tissues were homogenized in 0.25 M sucrose solution containing 20 mM Tris-HCl, pH 7.5, 1 mM EGTA, 1 mM EDTA, and 1 mM dithiothreitol plus protease inhibitors as described (16). After cell debris and nuclei were removed by a 10-min centrifugation at 1,000 g, the membrane/microsomal fractions were obtained by 30 min of centrifugation at 10,000 g. The membrane/microsomal fractions were solubilized in 0.25 M sucrose solution containing 0.1% Triton X-100. An aliquot (100 μg protein) of the extracts was subjected to eNOS assay. The eNOS activity was measured by monitoring the conversion of [14C]arginine to [14C]citrulline as described (16). The standard reaction mixture for the eNOS assay contained 50 mM HEPES buffer, pH 7.5, 10 mM MgCl2, 1 mM CaCl2, 100 mM calmodulin, 1 mg/ml...
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BSA, 1 mM dithiothreitol, 1 mM NADPH, 10 μM FAD, 10 μM tetrahydrobiopterin, and 1 μM [3H]arginine in a final volume 100 μl. After 20-min incubation at 30°C, the reaction was terminated with 200 μl of 100 mM HEPES buffer, pH 5.5, and 10 mM EDTA. After application to 0.5-ml columns of Dowex AG50WX-8 (Na⁺ form), [3H]citrulline was eluted with 1 ml water and quantified in a liquid scintillation counter. More than 90% of NOS activity in the total heart tissue was recovered in the membrane/microsomal fraction under this condition.

**Isoproterenol administration.** Vehicle (saline) or DL-isoproterenol (10 and 5 mg/kg; Sigma) in saline was injected intraperitoneally in a volume of 0.1 ml/100 g body wt once a day for 28 days in the sham (n = 8 for 5 mg/kg and n = 6 for 10 mg/kg), PO (n = 7), OVX (n = 6), and OVX-PO (n = 6) groups.

**Statistical analysis.** Values are represented as means ± SE. Infarct size and SDS-PAGE results were evaluated for differences by one-way ANOVA combined with Dunnett’s post hoc test. Survival was analyzed by Kaplan-Meier analysis. A value of P < 0.05 was considered statistically significant.

**RESULTS**

**Morphometric measurements.** Averages of serum estrogen level, heart (including both left and right ventricle), lung, and body weights (BW) for sham, OVX, PO, and OVX-PO groups are presented in Table 1. Serum estrogen level was significantly decreased following ovariectomy compared with the sham rats (sham: 56.6 ± 8.3 pg/ml; OVX: 3.6 ± 2.3 pg/ml, P < 0.01 compared with sham). Pressure load-induced hypertrophy had no effect on the serum estrogen level as seen both in the PO (60.8 ± 13.6 pg/ml, not significant compared with sham) and OVX-PO (3.4 ± 1.2 pg/ml, not significant compared with OVX) group rats. Following ovariectomy, BW (P < 0.001), heart weights (HW; P < 0.05), and lung weights (LW; P < 0.01) were significantly increased in the OVX rats compared with sham rats. Significant increases in LV weight were also seen in the OVX-PO group (P < 0.01 vs. OVX) compared with the OVX group, without significant changes in right ventricle weight. Significant increases in LV weight were also observed in the OVX-PO group (P < 0.05 vs. OVX) compared with the OVX group. LV weights (P < 0.01) and LWs (P < 0.001) were also significantly increased in the PO group compared with sham animals. Heart rate was only significantly increased in the OVX-PO group (P < 0.05 vs. sham) compared with sham rats. Notably, the HW-to-BW ratio was markedly increased both in the OVX-PO group (P < 0.001 vs. OVX) compared with the OVX group and in the PO group (P < 0.001 vs. sham) compared with sham (Fig. 1A). Increases in the LW-to-BW ratio were also significant both in the OVX-PO (P < 0.001) compared with OVX group and in the PO group (P < 0.01) compared with the sham group (Fig. 1B).

**Hemodynamic measurements.** Mean arterial blood pressure (MABP) was significantly increased in OVX-PO rats (P < 0.05) compared with OVX rats (Fig. 2A). Left ventricular end diastolic pressure (LVEDP) was more significantly increased in the OVX-PO group (P < 0.001) compared with the OVX group. Similarly, left ventricular developed pressure (LVDP) was significantly increased in the OVX-PO group (P < 0.001) compared with the OVX group (Fig. 2C). The rate of LV contraction (+dP/dt) and relaxation (−dP/dt) was also significantly increased in OVX-PO group (P < 0.001) compared with the OVX group. Reduced expression of eNOS was also observed in the PO group (P < 0.01 vs. sham) compared with sham. A significant negative linear correlation was observed between the HW-to-BW ratio and LV eNOS expression (Fig. 3B) and between LW/BW and LV eNOS expression (Fig. 3C). We also measured the eNOS activity in the LVs of the sham, OVX, PO, and OVX-PO rats. Similar with the reduced eNOS protein expression, we also found a significant reduction of eNOS activity in the OVX-PO group (OVX-PO: 0.12 ± 0.03 vs. sham: 0.25 ± 0.02 or vs. OVX: 0.20 ± 0.01 pmol·mg⁻¹·min⁻¹, P < 0.05) compared with sham and OVX groups. The eNOS activity was also significantly reduced in the PO group (PO: 0.19 ± 0.01 vs. sham: 0.25 ± 0.02 pmol·mg⁻¹·min⁻¹, P < 0.05) compared with sham rats. Expression of nNOS (P < 0.05 vs. OVX) and iNOS (P < 0.01 OVX) was also significantly decreased only in the OVX-PO group compared with the OVX group (Fig. 3A). Taken together, these observations show that ovariectomy alone has no effect on the eNOS, nNOS, and iNOS expression in the heart. However, pressure overload stress severely impaired eNOS, nNOS, and iNOS expression in the OVX-PO rat hearts and only eNOS expression in the PO rat hearts.

**Effects of pressure overload and ovariectomy on LV eNOS, nNOS, and iNOS expression.** To define the influence of ovarian hormone deprivation on cardiac NOS expression, we determined the effect of ovariectomy and pressure overload on expression of eNOS, nNOS, and iNOS in the female rat heart (Fig. 3A). Immunoblot analyses of heart lysates showed marked reduction of eNOS expression in the OVX-PO group (P < 0.001 vs. OVX) compared with the OVX group. Reduced expression of eNOS was also observed in the PO group (P < 0.01 vs. sham) compared with sham. A significant negative linear correlation was observed between the HW-to-BW ratio and LV eNOS expression (Fig. 3B) and between LW/BW and LV eNOS expression (Fig. 3C). We also measured the eNOS activity in the LVs of the sham, OVX, PO, and OVX-PO rats. Similar with the reduced eNOS protein expression, we also found a significant reduction of eNOS activity in the OVX-PO group (OVX-PO: 0.12 ± 0.03 vs. sham: 0.25 ± 0.02 or vs. OVX: 0.20 ± 0.01 pmol·mg⁻¹·min⁻¹, P < 0.05) compared with sham and OVX groups. The eNOS activity was also significantly reduced in the PO group (PO: 0.19 ± 0.01 vs. sham: 0.25 ± 0.02 pmol·mg⁻¹·min⁻¹, P < 0.05) compared with sham rats. Expression of nNOS (P < 0.05 vs. OVX) and iNOS (P < 0.01 OVX) was also significantly decreased only in the OVX-PO group compared with the OVX group (Fig. 3A). Taken together, these observations show that ovariectomy alone has no effect on the eNOS, nNOS, and iNOS expression in the heart. However, pressure overload stress severely impaired eNOS, nNOS, and iNOS expression in the OVX-PO rat hearts and only eNOS expression in the PO rat hearts.

**Effects of pressure overload and ovariectomy on LV Akt and p38-MAPK phosphorylation.** To examine the role of Akt signaling in sex-mediated cardioprotection against myocardial hypertrophy, Akt phosphorylation both at Ser⁴73 and Thr⁴⁰⁶, which is essential for Akt activity, was examined in cell lysates
from sham, OVX, PO, and OVX-PO rat hearts. Akt phosphorylation at Ser473 was significantly reduced in the hearts of OVX-PO rats (P < 0.01 vs. OVX) compared with the OVX group (Fig. 4A), indicating that Akt signaling is markedly impaired in the OVX-PO heart concomitant with severe cardiac hypertrophy, as seen in Fig. 1 and Table 1. Akt Thr308 phosphorylation also decreases only in the OVX-PO group (Fig. 4A), but the change was not significant compared with the OVX group. On the contrary, there were no significant changes in the total Akt protein (P = not significant) in sham, OVX, PO, and OVX-PO rat hearts.

Because p38-MAPK signaling is activated in cardiomyocytes by several hypertrophic stressors (6, 42), we examined p38-MAPK phosphorylation in the hypertrophic heart under various conditions. OVX-PO (P < 0.01 vs. sham) and PO (P < 0.05 vs. sham) groups showed significant increases in p38-MAPK phosphorylation compared with sham animals without changes in total p38-MAPK protein (Fig. 4B).

**Effects of pressure overload and ovariectomy on LV MLC phosphorylation.** Because phosphorylation of MLC at Ser19 reportedly correlates with increased maximum tension or dP/dt values in an intact heart (41), we examined MLC phosphorylation...
We found a significant and marked increase in MLC phosphorylation (Ser19) in the OVX-PO group (\(P < 0.001\) vs. OVX). Total MLC content was also significantly decreased in the OVX-PO group (\(P < 0.01\) vs. OVX; Fig. 4C). Taken together, the ratio of the phospho-MLC to total MLC was significantly and markedly elevated only in the OVX-PO group (\(P < 0.001\) vs. OVX) compared with the other three groups (Fig. 4C).

Effects of pressure overload and ovariectomy on LV HSPs and caveolin-3 expression. We determined the regulation of HSP-70 and HSP-90 by ovariectomy and pressure overload in the LV of sham, OVX, PO, and OVX-PO rats. Ovariectomy significantly elevated HSP-90 (\(P < 0.01\) vs. sham) and HSP-70 (\(P < 0.05\) vs. sham) protein concentrations in LV cell lysates of the OVX rats (Fig. 5, A and B). We also found a significant reduction of HSP-90 (\(P < 0.01\) vs. OVX) and HSP-70 (\(P < 0.01\) vs. OVX) protein expression in the OVX-PO group compared with the OVX rats (Fig. 5, A and B). We also measured the effect of ovariectomy and pressure overload on the expression of NOS regulatory protein caveolin-3 in the LV of sham, OVX, PO, and OVX-PO rats. Ovariectomy significantly elevated caveolin-3 expression (\(P < 0.01\) vs. sham) in the LV cell lysates of the OVX rats (Fig. 5C). These findings confirm that ovariectomy induces HSP-70, HSP-90, and caveolin-3, whereas pressure overload decreases their expression in the hearts of the ovariectomized rats.

Effects of pressure overload and ovariectomy on LV dystrophin levels. Dystrophin is essential to maintain membrane integrity and mechanical strength in cardiomyocytes; however, little is known about how dystrophin levels in the heart are regulated in response to estrogen and pressure overload. Western blotting analysis using heart cell lysates showed that 400-kDa dystrophin levels were markedly reduced in the OVX group (\(P < 0.001\) vs. sham), PO group (\(P < 0.001\) vs. sham), and OVX-PO group (\(P < 0.001\) vs. OVX) (Fig. 6), indicating that dystrophin breakdown follows both ovariectomy and pressure overload.

Effects of chronic \(\beta\)-adrenergic stimulation by isoproterenol on rat survival. To test effects of chronic \(\beta\)-adrenergic stimulation following estrogen depletion and pressure overload, mortality rate curves were examined after chronic treatment with isoproterenol in sham, OVX, PO, and OVX-PO rats. Kaplan-Meier survival plots are shown in Fig. 7. Chronic treatment with isoproterenol (5 mg/kg for 28 days) had no effect on sham and PO rats, but treatment of sham rats with 10 mg/kg increased death with a survival rate of 67% at 28 days (Fig. 7). Remarkably, ovariectomy tended to increase mortal-
ity, with a survival rate of 84% at 28 days in OVX plus isoproterenol (5 mg/kg; \( P < 0.001 \) vs. all groups). The most significant mortality was observed in the OVX-PO rats, with a survival rate of 0% at 21 days following isoproterenol (5 mg/kg) treatment. These observations indicate that decompensation against an acute cardiac stress such as isoproterenol administration occurs only in OVX and OVX-PO animals.

**DISCUSSION**

The incidence of cardiovascular disease increases in postmenopausal women, but little is known about potential negative effects of menopause on cardiac function and remodeling. To examine cardiac compensatory phenotypes mediated by ovariectomy against pressure overload stress, female rats were subjected to abdominal aortic stenosis with or without bilateral ovariectomy. Notably, the HW-to-BW ratio was most significantly increased both in the OVX-PO group compared with the OVX group and in the PO group compared with sham. Similarly, the LW-to-BW ratio was also significantly increased both in the OVX-PO group compared with the OVX group and in the PO group compared with the sham group. These findings indicate that pressure overload but not ovariectomy is the causative stress for increased HW. However, the most significant and marked increases in LVEDP, LVDP, and \( \frac{d}{dt}_{\text{max}} \) were observed only in the OVX-PO group compared with the OVX group. Therefore, our aim was to define mechanisms involved in detrimental effects of ovariectomy in the female heart during development of myocardial hypertrophy.

Ovarian hormones are believed to possess cardiovascular protective effects, and they seem to play a role in the sex-related differences in the development of hypertension in experimental models (3, 7). Naturally, ovariectomy causes a significant reduction in estradiol and progesterone levels (9, 21). Comparison of estradiol levels with MABP in Dahl salt-sensitive (DS) rats suggests that the OVX-induced increase in MABP is associated with decreased levels of plasma estrogen because estradiol replacement was able to prevent the OVX-induced hypertension (21). Moreover, estradiol supplementation did not markedly alter circulating progesterone levels in either young or aged DS rats (21). Furthermore, progesterone levels did not correlate with the difference in MABP.

![Effects of pressure overload and ovariectomy on Akt, p38-mitogen-activated protein kinase (MAPK) and MLC phosphorylation in the LV. A: top, representative image showing Western blots using cell extracts from sham, OVX, PO, and OVX-PO hearts probed with specific antibodies against phosphorylated protein kinase B (Akt) Ser\(^{473} \), phosphorylated Akt Thr\(^{308} \), and total Akt. Bottom, quantitative analyses of phospho-Akt Ser\(^{473} \), phospho-Akt Thr\(^{308} \), and total Akt levels were determined by densitometric analyses of the Western blots. B: top, representative image showing Western blots using cell extracts from sham, OVX, PO, and OVX-PO hearts probed with specific antibodies against phosphorylated p38 MAPK Thr\(^{180}/\text{Tyr}^{182} \) and total p38 MAPK. Bottom, quantitative analyses of p38 MAPK Thr\(^{180}/\text{Tyr}^{182} \) and total p38 MAPK levels were determined by densitometric analyses of Western blots. C: top, representative image showing Western blots using cell extracts from sham, OVX, PO, and OVX-PO hearts probed with a specific antibody recognizing phosphorylated myosin light chain Ser\(^{19} \) and total myosin light chain 2. Bottom, quantitative analysis of the ratio of phosphorylated myosin light chain light to total myosin light chain was performed by densitometric analysis of blots. Data are expressed as percentages of the value of sham-operated rats. Each bar represents the mean ± SE for 5 experiments. *\( P < 0.05 \), **\( P < 0.01 \), and ***\( P < 0.001 \) vs. the sham-operated group; ††\( P < 0.01 \) and †††\( P < 0.001 \) vs. the OVX group; ##\( P < 0.01 \) and ###\( P < 0.001 \) vs. the PO group.
between the OVX and OVX-estrogen groups in DS rats (21). This lack of effect of progesterone supports previous reports in deoxycorticosterone salt hypertension showing that progesterone had no effect on the development of hypertension in OVX rats (7).

Potentiation of NO synthesis activities via activation of eNOS, nNOS, and iNOS by estrogen has been documented as a beneficial mechanism underlying estrogen-induced cardiovascular protection. For example, estrogen treatment upregu-
lates both eNOS (47) and iNOS (17) in cardiomyocytes. Under physiological conditions, estrogen-induced iNOS upregulation also provides a cardiovascular protective effect (15, 28). Furthermore, iNOS upregulation is associated with prevention of platelet aggregation, which causes vascular injury (19). However, the role of estrogen-induced upregulation of NOs has not been determined under pressure overload-induced stress in female hearts in vivo. We found that ovariectomy alone has no effect on the eNOS, iNOS, and nNOS expression in the heart. However, pressure overload stress severely impaired eNOS, iNOS, and nNOS expression in the OVX-PO rat hearts and only eNOS expression in the PO rat hearts. The most pronounced reduction in expression of all three NOs isoforms, particularly in eNOS, was observed only in the OVX-PO group in which both systolic and diastolic dysfunction was observed, as shown in Fig. 2. Taken together, severe reduction in eNOS, nNOS, and iNOS levels likely mediates impaired systolic and diastolic dysfunction observed in PO rats following ovariectomy. Moreover, we also found significantly increased expression of the NOs regulatory proteins like HSP-90, HSP-70, and caveolin-3 in the hearts of the OVX rats, and pressure overload downregulated their expression on the OVX-PO rats.

Both the PI 3-kinase-Akt (22, 40) and ERK pathways (32) are downstream targets of nonnuclear estrogen signaling. Estrogen activates eNOS activity through Akt and ERK pathways, thereby promoting NO production in heart (40). Thus Akt activity is also essential for estrogen-induced eNOS upregulation. Interestingly, Akt phosphorylation at Ser173 is significantly reduced in hearts from OVX-PO rats compared with the OVX group, indicating that downregulation of Akt activity by OVX-PO significantly antagonizes the compensatory effect of eNOS. Thus simultaneous impairment of eNOS levels and Akt activity likely cause cardiac decompensation following ovariectomy. Notably, p38MAPK phosphorylation is also significantly increased in PO and OVX-PO groups compared with sham animals. Previously, van Eickels et al. (46) demonstrated that inhibition of p38MAPK phosphorylation by estrogen treatment accounts for the estrogen-induced anti-hypertrophic effect in pressure overload. Further study is required to define how p38MAPK causes detrimental effects on heart function caused by pressure overload.

Under physiological conditions, MLC phosphorylation correlates with increased maximum tension or dP/dt values (41). In the present study, we found that phosphorylation was markedly increased only in OVX-PO rats. By contrast, total MLC content was significantly decreased in OVX-PO rats. The increased MLC phosphorylation-to-total MLC ratio was likely associated in part with an increase in ±dP/dt_max in heart contractile function. NO in vascular smooth muscle activates soluble guanylyl cyclase to increase cGMP formation, thereby leading to a decrease in intracellular Ca^{2+} concentration with subsequent inhibition of MLC phosphorylation and contraction (35, 38, 39). Our data suggest that ovariectomy followed by pressure overload subsequently increases MLC phosphorylation accompanied by increased cardiac contractility, as observed particularly in OVX-PO rats.

Dystrophin abnormalities cause cardiomyopathy and skeletal muscle disorders such as muscular dystrophy. Significant cardiac damage, including myocyte injury, inflammation, and fibrosis, was seen in dystrophin-deficient myocardium during pressure overload (25). Moreover, the sarcomlemmal integrity of young female X-linked muscular dystrophy mice was greater than that seen in corresponding age-matched dystrophic males and ovariectomized females. This observation indicates that estrogen mediates protection against muscular dystrophy in female mice (37). However, the pathogenesis of dystrophin abnormalities following ovariectomy remains unclear. We observe downregulation of dystrophin in response to ovariectomy and cardiac hypertrophy. Therefore, dystrophin breakdown following ovariectomy and pressure overload likely mediates vulnerability of estrogen-deficient myocardium to pressure overload.

β-Adrenoceptor (β-AR) agonists differently affect heart rate during the estrous cycle in female rats and in ovariectomized rats with or without estrogen replacement (5). Ovariectomy also increases susceptibility to the effects of β-AR agonists (44). Importantly, Kam et al. (24) demonstrated that β-AR stimulation with isoproterenol led to a significantly greater increase in electrical stimulation-induced Ca^{2+} elevation, Ca^{2+} uptake through cardiac L-type Ca^{2+} channels, heart rate, and contractility in hearts of ovariectomized rats compared with sham rats. These responses were rescued by estrogen replacement. Interestingly, Kaplan-Meier survival data in our study clearly indicates that significantly increased mortality occurs only in the OVX-PO group following chronic β-adrenergic stimulation by isoproterenol (5 mg/kg). No death was observed in sham and PO groups treated with low doses (5 mg/kg) of isoproterenol. Thus cardiac decompensation by ovariectomy causes increased mortality during chronic β-adrenergic stimulation.

In conclusion, the most significant observation presented here is that simultaneous severe reduction of eNOS and Akt activity in pressure overload stress with ovariectomy likely mediates increased compensatory effects on heart contractility in cardiac hypertrophy. In addition to decreased eNOS levels and Akt activity, marked breakdown of dystrophin by both ovariectomy and pressure overload likely contributes to increased mortality following acute cardiac stress caused by chronic β-adrenergic stimulation. Ovariectomy likely accounts for cardiac decompensation against chronic stress through impairment of functions of NOs and Akt signaling.

GRANTS

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