Prevention of adverse cardiac remodeling to volume overload in female rats is the result of an estrogen-altered mast cell phenotype

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Lu H, Meléndez GC, Levick SP, Janicki JS. Prevention of adverse cardiac remodeling to volume overload in female rats is the result of an estrogen-altered mast cell phenotype. Am J Physiol Heart Circ Physiol 302: H811–H817, 2012. First published December 9, 2011; doi:10.1152/ajpheart.00980.2011.—Previously, we have reported sex differences in the cardiac remodeling response to ventricular volume overload whereby male and ovariectomized (OVX) female rats develop eccentric hypertrophy, and intact (Int) female rats develop concentric hypertrophy. In males, this adverse remodeling has been attributed to an initial cascade of events involving myocardial mast cell and matrix metalloproteinase activation and extracellular collagen matrix degradation. The objective of this study was to determine the effect of female hormones on this initial cascade. Accordingly, an aortocaval fistula (Fist) was created in 7-wk-old Int and OVX rats, which, together with sham-operated (sham) controls, were studied at 1, 3, and 5 days postsurgery. In Int-Fist rats, myocardial mast cell density, collagen volume fraction, endothelin (ET)-1, stem cell factor (SCF), and TNF-α remained at control levels or were minimally elevated throughout the study period. This was not the case in the OVX-Fist group, where the initial response included significant increases in mast cell density, collagen degradation, ET-1, SCF, and TNF-α. These events in the OVX-Fist group were abolished by pretreatment with a mast cell stabilizer nedocromil. Of note was the observation that ET-1, TNF-α, SCF, and collagen volume fraction values for the OVX-sham group were greater than those of the Int-sham group, suggesting that the reduction of female hormones alone results in major myocardial changes. We concluded that female hormone-related cardioprotection to the volume stressed myocardium is the result of an altered mast cell phenotype and/or the prevention of mast cell activation.

extracellular collagen; tumor necrosis factor-α; female hormones; stem cell factor; endothelin-1; nedocromil

WHILE THE INCREASED RISK OF CARDIOVASCULAR DISEASE AFTER MENOPAUSE IN WOMEN HAS HISTORICALLY BEEN ATTRIBUTED TO THE ACCOMPANYING SEX HORMONAL CHANGES, THE MECHANISMS RESPONSIBLE FOR THE CARDIOPROTECTION IN PREMENOPAUSAL WOMEN HAVE NOT BEEN ELUCIDATED. RECENT STUDIES FROM OUR LABORATORY HAVE FOUND SEX DIFFERENCES IN THE PATTERN OF GLOBAL MYOCARDIAL REMODELING. WHILE MALES DEVELOP ECCENTRIC HYPERTROPHY AND HEART FAILURE IN RESPONSE TO A SUSTAINED VENTRICULAR VOLUME OVERLOAD, FEMALES DEVELOP CONCENTRIC HYPERTROPHY AND THE HEART REMAINS COMPENSATED (12). AS A RESULT, THERE IS A HIGHER MORTALITY RATE IN MALES (24.5%) AFTER 8 WK OF VOLUME OVERLOAD COMPARED WITH ONLY 2.5% IN FEMALES (12). THE ADVERSE REMODELING IN MALES IS INITIATED BY THE DEGRANULATION OF CARDIAC MAST CELLS SECONDARY TO AN INCREASE IN ENDOTHELIN (ET)-1 THAT RESULTS IN 1) A SIGNIFICANT INCREASE IN MAST CELL DENSITY, 2) AN INCREASE IN THE LEVELS OF MYOCARDIAL TNF-α, AND 3) THE SUBSEQUENT SIGNIFICANT DEGRADATION OF MYOCARDIAL FIBRILLAR COLLAGEN (2, 22). BECAUSE THESE EVENTS ARE SEQUENTIAL, IT IS NOT KNOWN WHICH ARE EFFICACIOUSLY AFFECTED BY FEMALE HORMONES. IN ADDITION, UPLIFTED LEVELS OF STEM CELL FACTOR (SCF) HAVE BEEN SHOWN TO INCREASE CARDIAC MAST CELL DENSITY (25). THEREFORE, WE HYPOTHESIZED THAT FEMALE HORMONE-RELATED CARDIOPROTECTION OF THE VOLUME STRESSED MYOCARDIUM IS THE RESULT OF AN ESTROGEN-ALTED MAST CELL PHENOTYPE AND/OR AN ESTROGEN-RELATED PREVENTION OF MAST CELL ACTIVATION.

To test this hypothesis, an aortocaval fistula was created in 7-wk-old intact (Int) and ovariectomized (OVX) rats, which, together with sham-operated (sham) controls, were studied at 1, 3, and 5 days postsurgery. We found that female rats with intact ovaries had no change in mast cell density as well as no subsequent changes in myocardial SCF and TNF-α levels and collagen degradation. In contrast, OVX rats had increased mast cell density, increased myocardial SCF and TNF-α levels, and collagen degradation that was prevented by mast cell stabilization.

METHODS

Animals. All of the animal experiments conformed with the principles of the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, and the protocols were approved by our institutional Animal Care and Use Committee. Experiments were performed with 7- or 8-wk-old Sprague-Dawley female rats purchased from Harlan Laboratories. All animals were housed under standard environmental conditions and maintained on a normal rodent diet not containing alfalfa or soybean meal (diet no. 2014, Harlan Teklad) and tap water ad libitum. Anesthesia for nonterminal surgical procedures was achieved using inhaled isoflurane (4%), with postoperative analgesia maintained by the administration of buprenorphine hydrochloride (0.025 mg/kg). Euthanization was performed by removal of the heart after rats had been deeply anesthetized by an intraperitoneal injection of pentobarbital sodium (70 mg/kg).

Surgical procedures and experimental protocol. Volume overload was induced by aortocaval fistula as previously described (12). Briefly, after a ventral laparotomy to expose the abdominal aorta and the caudal side of the inferior vena cava, an 18-gauge needle was inserted into the infrarenal aorta (2 cm below the right renal artery) and advanced through the medial wall into the vena cava. The control (sham) group consisted of female rats who were subjected to the laparotomy surgery without the creation of a fistula. Ovariectomies were performed 1 wk before the fistula or sham procedure. The ovarian pedicles were ligated, and the ovaries were completely excised, as previously described (4). Rats were randomly assigned to one of the following groups: fistulas with intact ovaries (Int-Fist) and their respective shams (Int-Sham) (study end points: 1, 3, and 5 days postsurgery); ovariectomy + fistula (OVX-Fist) with their respective
ET-1, TNF-α, H9252, and SCF as well as plasma 17β-estradiol (OVX-sham) (study end points: 1, 3, and 5 days postsurgery); and ovariectomy + fistula + nedocromil (OVX-Fist-Ned) (study end points: 3 and 5 days postsurgery). For the OVX-Fist-Ned group, the nedocromil pellet (release rate: 30 mg·kg⁻¹·day⁻¹, Innovative Research of America) was implanted subcutaneously immediately after ovariectomy (1 wk before the fistula). Each group at each time point consisted of five to eight rats. At the experimental end point, body weight was recorded, a patent fistula was confirmed by the presence of pulsatile flow of oxygenated blood into the vena cava, venous blood was collected in EDTA-containing tubes, and the heart and uterus were removed. The uterus as well as the right ventricle (RV) and left ventricle (LV) of the heart and the lungs were weighed. A midventricular, transmural LV section was placed in 4% paraformaldehyde for paraffin embedding and histological analysis. The apex was snap frozen in liquid nitrogen and stored at −80°C for further analysis. The lungs were separated from the esophagus and trachea and weighed after excess moisture was blotted from the pleural surfaces.

**Measurements of myocardial mast cell density.** Paraformaldehyde-fixed tissue was paraffin blocked, and 5-μm serial sections were stained with toluidine blue as previously described (2). Mast cell density was determined by dividing the total number of mast cells per LV cross section by the tissue area of the section.

**Measurements of collagen volume fraction in the rat myocardium.** Serial sections (5 μm) were stained with collagen-specific picrosirius red (0.1% Sirius red F3BA in picric acid) after an incubation in phosphomolybdic acid (0.2%). Twenty random fields per section, with exclusion of the perivascular regions, were analyzed using a Zeiss Axiosvert 200 fluorescent microscope (magnification: ×200) and analyzed with ImageJ software (NIH) as previously described (20).

**ELISA analysis of ET-1, TNF-α, SCF, and 17β-estradiol.** Approximately 100 mg of LV tissue were homogenized with a protease inhibitor cocktail (P8340, Sigma-Aldrich). Plasma was collected from the inferior vena cava with 1.8 mg/ml EDTA. Myocardial levels of ET-1, TNF-α, and SCF as well as plasma 17β-estradiol concentrations were determined using the following commercially available ELISA kits: ET-1 (Alpco Diagnostics), TNF-α (BD Biosciences), SCF (Abcam); and 17β-estradiol (Calbiotech). All samples were run in duplicate and averaged.

<table>
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<th>Group</th>
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<th>Body Weight, g</th>
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<th>Right Ventricular Weight, mg</th>
<th>Lung Weight, mg</th>
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<td></td>
<td></td>
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<td>161 ± 9*</td>
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Values are means ± SD; n, number of rats. Int, intact ovaries; sham, sham operation; Fist, aortocaval fistula surgery; OVX, ovariectomy; Ned, nedocromil treatment. *P < 0.05 vs. the Int-sham group; †P < 0.05 vs. the OVX-sham group.

Fig. 1. Left ventricular (LV) mast cell density in female rats with intact ovaries (A) and after ovariectomy (B). The following groups are shown: rats with intact ovaries subjected to sham operation (Int-Sham) and with intact ovaries subjected to fistula surgery (Int-Fist) as well as ovariectomized (OVX) rats subjected to sham operation (OVX-sham) or fistula surgery (OVX-Fist). All values are means ± SD. *P < 0.001 vs. the OVX-sham group.
**Statistical analysis.** Data were analyzed using SPSS 11.5 and SigmaPlot (version 11) software. To compare the values between two study groups, we used Student’s t-test, and to compare more than two groups, we used one-way ANOVA. Data are represented as means ± SD or SE as indicated in the RESULTS. P values of <0.05 were considered statistically significant.

**RESULTS**

**Biometric parameters.** Body, LV, RV, and lung weights for all experimental groups are shown in Table 1. There were no significant differences in body and lung weights between any fistula group and their corresponding shams at any of the time points. Volume overload did not lead to any change of LV weight in rats with intact ovaries, whereas LV weight in O VX rats increased as early as 3 days after fistula and persisted through to 5 days. RV weight was significantly increased at 3 and 5 days after fistula in Int rats and at 3 days in O VX rats. Nedocromil treatment attenuated the increase in LV weight in O VX rats. Uterus weight was significantly decreased in O VX animals as an indicator of successful removal of the ovaries (353 ± 28 and 208 ± 18 mg, P < 0.001). In addition, O VX rats had significantly lower plasma 17β-estradiol concentrations compared with those with intact ovaries (intact ovaries vs. ovariectomy: 27.8 ± 4.7 vs. 15.7 ± 1.3 pg/ml, P < 0.01).

Volume overload led to increases of mast cell density and collagen degradation in LVs of O VX rats. The temporal responses postfistula of LV mast cell density are shown in Fig. 1. Volume overload had no effect on LV mast cell density in Int female rats (Fig. 1A). In contrast, mast cell density was significantly increased by nearly 50% 3 days after fistula in O VX female rats (Fig. 1B) and returned to normal 5 days postfistula. While there was a tendency for mast cell densities to be slightly greater in the Int-sham groups compared with the O VX-sham groups, the differences were not statistically different.

The postfistula response in LV collagen volume fraction is shown in Fig. 2. Consistent with the findings in mast cell density, volume overload did not change collagen volume fraction in female rats with intact ovaries (Fig. 2A). Volume overload did not change collagen volume fraction in O VX rats at 1 or 3 days after fistula but did significantly decrease collagen volume fraction at 5 days after fistula. As was the case with mast cell density, the collagen volume fraction values for the Int-sham groups at 3 and 5 days were slightly greater than those for the O VX groups. At 1 day, however, the O VX sham collagen volume fraction value was statistically greater than that in the Int-sham value. In contrast, at 5 days, collagen volume fraction value in the O VX-sham group was much less than that in the Int-sham group.

Volume overload resulted in increases in ET-1 concentrations in the LV. ET-1 concentrations in the LV were measured using a commercial ELISA kit, and the results are shown in Fig. 3. Volume overload increased ET-1 concentrations 3 days after fistula surgery in rats with (Fig. 3A) and without (Fig. 3B) ovaries. However, the ET-1 value was 72% greater in O VX rats than that in Int-Fist rats. By 5 days after fistula, ET-1 had returned to normal levels in both groups. It should also be noted that, at all three postfistula time points, ET-1 values for

![Fig. 2. LV collagen volume fraction in female rats with intact ovaries (A) and after ovariectomy (B). All values are mean ± SE. *P < 0.05 vs. the O VX-sham group; †P < 0.05 vs. the relative Int group.](http://ajpheart.physiology.org/)

![Fig. 3. LV myocardial endothelin (ET)-1 concentration in female rats with intact ovaries (A) and after ovariectomy (B). All values are means ± SE. *P < 0.001 vs. the corresponding sham group; †P < 0.05 vs. the relative Int group.](http://ajpheart.physiology.org/)
the OVX-sham groups were statistically greater than those in the Int-sham groups.

**Volume overload led to increases in SCF concentrations in the LV.** LV myocardial SCF concentration results, measured using a commercial ELISA kit, are shown in Fig. 4. Volume overload increased SCF concentrations 3 and 5 days after fistula surgery in rats with (Fig. 4A) and without (Fig. 4B) ovaries. However, consistent with the findings of ET-1, the values attained were 1.7-fold greater in OVX rats. Also, the OVX-sham SCF value was significantly greater than that in the Int-sham group.

**Nedocromil ablated volume overload-induced phenotypic changes.** Mast cell stabilization prevented the increase in LV mast cell density (3 days after fistula; Fig. 5A) and reduction in collagen volume fraction (5 days after fistula; Fig. 5B) that occurred in nontreated OVX rats.

**TNF-α levels.** There were no changes in LV TNF-α between sham and fistula groups at any time point in rats with intact ovaries (Fig. 6A). OVX rats had a twofold increase in their LV TNF-α levels at both 3 and 5 days after fistula surgery compared with OVX-sham rats; nedocromil prevented this increase (Fig. 6B). Also, the OVX-Fist values, including those of the sham group, were two to four times statistically greater than those for the Int-Fist groups.

**DISCUSSION**

Clinical studies (8, 15, 24) have identified human menopause as a risk factor for the development of cardiovascular disease. Similarly, sex differences in cardiac remodeling have been shown in several animal models of heart failure, including experimentally induced pressure overload (9, 26, 27), spontaneously hypertensive rats (11), and myocardial infarction (18, 23). Using a model of chronic cardiac volume overload, our laboratory has convincingly identified sex differences in the progressive pattern of adverse myocardial remodeling and the development of heart failure. In response to a sustained elevation in cardiac volume, male rats rapidly developed LV wall thinning and dilatation and congestive heart failure, whereas female rats maintained a normal LV mass-to-volume ratio and remained compensated (12). Moreover, we reported that this cardioprotection was abolished by ovariectomy (4) but could be restored with supplemented estrogen (13), thus highlighting the crucial role of ovarian hormones in preventing adverse remodeling and preserving cardiac function. During the first week of volume overload, the subsequent adverse remodeling in males is initiated by an increase in ET-1 that then results in a significant increase in mast cell activation and density leading to matrix metalloproteinase activation and a marked degradation of myocardial fibrillar collagen (2). These initial events and subsequent adverse remodeling did not occur in mast cell-deficient rats (17) and when mast cell degranulation was pharmacologically prevented (2, 5). Although we have proven
that, in this model of chronic volume overload, estrogen is a vital hormone responsible for cardioprotection, it is unknown which of these initial events are efficaciously affected by female hormones.

The findings reported herein indicated that, in the intact female, both cardiac mast cell densities and collagen volume fractions remained at control levels throughout the duration of the study period. This was not the case in OVX rats, where instead the initial response included a significant increase in mast cell density and subsequent collagen degradation similar to that in males. Also similar to findings in males, nedocromil prevented these adverse postfistula events, indicating a central causative role for mast cells in provoking this adverse remodeling process. In addition, these results confirm the findings of Chancey et al. (6) regarding estrogen’s ability to efficaciously alter the phenotype of the mast cell. In that study, we found chemical degranulation of over 95% of the cardiac mast cells in isolated hearts from Int females and OVX rats given supplemental estrogen did not result in the activation of matrix metalloproteinases and collagen degradation that occurred in isolated hearts from OVX rats. Therefore, our findings further demonstrate that estrogen alters the phenotype of the cardiac mast cell and/or prevents degranulation to effectively inhibit the initial events that result in adverse cardiac remodeling (Fig. 7).

While the stimulus for the increase in mast cell density remains to be determined, we have shown that it too is associated with mast cell activation (3). Furthermore, it is conceivable that the fistula-induced increases in the levels of myocardial SCF that occurred in the OVX group, which were much greater than those in the Int group, were responsible for the increased mast cell density in this group. In view of the findings of Forman et al. (10), which indicated a significant increase in the ratio of mature to immature cardiac mast cells after 24 h of cardiac volume overload, one could surmise that the increased mast cell density was related to such a maturation process secondary to the elevated SCF levels. This is further supported by the findings of Tsai et al. (25), who have shown that the administration of SCF to primates twice daily for 21 days resulted in a significant increase in cardiac mast cell density and that 15 days after discontinuation of its administration, mast cell density was found to be normal.

Several studies (1, 16) have established that proinflammatory cytokines, such as TNF-α, contribute to the progression of the disease in male rats. Bozkurt et al. (1) were the first to report LV wall thinning, dilatation, and collagen degradation after the infusion of pathophysiological levels of TNF-α. Chen et al. (7) demonstrated that the stretch of volume overload induces the production of TNF-α, which triggers a strong inflammatory myocardial response, thus mediating adverse remodeling. Furthermore, we and others (14, 17) have demonstrated that cardiac mast cells are an important source of TNF-α. Having demonstrated that cardiac mast cell degranulation occurred in OVX rats after volume overload, we examined LV myocardial TNF-α levels after the creation of a fistula. While there was no increase in the Int group, there was a sustained twofold increase in TNF-α levels in OVX rats at 3 and 5 days postfistula relative to their respective sham groups. The levels attained in the OVX group postfistula were three- to
fourfold greater than those in the Int group. Furthermore, the mast cell stabilizer nedocromil prevented these increases, providing additional support to the observation that mast cells contribute to TNF-α synthesis (Fig. 7).

While the results reported herein and those of others indicate that the mast cell phenotype is altered by estrogen, it is also possible that upstream activators of mast cells are downregulated in the presence of estrogen, thereby preventing mast cell activation (Fig. 7). Recently, we (21) have reported in male rats the occurrence of significant elevations in LV myocardial ET-1 levels during the first week postfistula to be responsible for cardiac mast cell activation. The findings of the present study indicate that LV ET-1 was increased at 3 days postfistula in both groups of rats with and without ovaries, albeit to much lower levels than Murray et al. reported for males 1 day postfistula (i.e., 2.5–4.5 vs. 8–9 fmol/ml). Even though ET-1 was significantly elevated in rats with and without ovaries, OVX-Fist rats expressed a twofold greater increase in ET-1 levels compared with Int-Fist rats. Similarly, our previous study (22) in male rats documented a twofold increase in ET-1 levels at 3 days postfistula. Hence, it is possible that levels of ET-1 have to exceed a threshold value to induce mast cell activation, explaining why activation of mast cells did not occur in females with intact ovaries and aortocaval fistula. Estrogen may be an important regulator of ET-1; however, the exact regulatory mechanism of this upstream pathway leading to mast cell degranulation is yet to be elucidated. Similarly, the mechanism underlying the return of ET-1 to normal levels by 5 days postfistula is unknown. One possibility may be its degradation by excreted chymase from activated mast cells. Another possibility is upregulation of the ET-1 clearance receptor. However, without knowing the source and/or stimulus for the increase in ET-1, its transient response is difficult to explain. Obviously, further research is required.

Finally, the fact that ET-1, TNF-α, SCF, and collagen volume fraction values for the OVX-sham group were greater than those of the Int-sham group is noteworthy and indicates that estrogen plays a role in maintaining basal levels of these parameters in female rats. In the fistula plus nedocromil group, mast cell stabilization was initiated at the time of ovariectomy and the ovariectomy-stimulated baseline changes were still present at the study end point. This infers that these increased values were mast cell independent. Thus, in addition to an altered mast cell phenotype, the decrease in estrogen after ovariectomy may have affected other estrogen-sensitive cells in heart that synthesize ET-1, TNF-α, SCF, and collagen. That this is the case for TNF-α was recently demonstrated in our laboratory (19), where we found both its myocardial mRNA and concentration to be significantly increased 3 days postfistula in males, whereas supplemental estrogen prevented these increases. Nevertheless, nedocromil prevented the postfistula changes in the OVX group, indicating a central role for the estrogen-deficient myocardial mast cell in the initial adverse remodeling secondary to a sustained increase in cardiac volume.

In summary, we demonstrated the critical role that ovarian estrogen plays in preventing the initial phase of adverse cardiac remodeling secondary to volume overload. We found that female rats with intact ovaries had no change in mast cell density as well as no subsequent changes in myocardial TNF-α levels and collagen degradation. In contrast, OVX rats had a similar phenotype as that in male rats, which was ablated by mast cell pharmacological stabilization. Thus, this study is the first to establish a mechanistic description of estrogen-mediated cardioprotection.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: H.L., G.C.M., and S.P.L. performed experiments; H.L. analyzed data; H.L., S.P.L., and J.S.J. interpreted results of experiments; H.L. and G.C.M. prepared figures; H.L. and G.C.M. drafted manuscript; H.L., G.C.M., S.P.L., and J.S.J. edited and revised manuscript; H.L., G.C.M., S.P.L., and J.S.J. approved final version of manuscript; S.P.L. and J.S.J. conception and design of research.

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