Modulation of cardiac mast cell-mediated extracellular matrix degradation by estrogen

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Chancey, Amanda L., Jason D. Gardner, David B. Murray, Gregory L. Brower, and Joseph S. Janicki. Modulation of cardiac mast cell-mediated extracellular matrix degradation by estrogen. Am J Physiol Heart Circ Physiol 289: H316–H321, 2005. First published February 18, 2005; doi:10.1152/ajpheart.00765.2004.—There are fundamental differences between males and females with regard to susceptibility to heart disease. Although numerous animal models of heart failure have demonstrated that premenopausal females are afforded cardioprotection and, therefore, fare better in the face of cardiac disease than their male counterparts, many questions as to how this occurs still exist. Recently, we showed that 1) increased mast cell density is associated with adverse ventricular remodeling and 2) chemically induced mast cell degranulation using compound 48/80 resulted in remarkable changes in matrix metalloproteinase (MMP) activity, cardiac collagen structure, and cardiac diastolic function in normal male rats. With the known gender differences in cardiac disease in mind, we sought to examine the effects of chemically induced cardiac mast cell matrix degradation in isolated, blood-perfused hearts of intact female rats, ovariectomized female rats, and ovariectomized female rats treated with 17β-estradiol. In response to mast cell degranulation, no significant differences in cardiac function, MMP-2 activity, or collagen volume fraction were observed between intact female rats and ovariectomized female rats treated with estrogen. In the ovariectomized female group, a significant upward shift in the left ventricular pressure-volume relation, accompanied by a marked 133% increase in active MMP-2 values over that in the intact female group, was noted after treatment with compound 48/80 (P < 0.05), along with a significant reduction in collagen volume fraction below control (0.46 ± 0.23 vs. 0.73 ± 0.13%, P < 0.05). These findings indicate that estrogen’s cardioprotective role can be partially mediated by its effects on cardiac mast cells, MMPs, and the extracellular matrix.

This finding was established by previous studies demonstrating an association between increased mast cell density, activation of MMPs, and initiation of cardiac remodeling in volume overload induced by an AV fistula in male rats (4, 22) and by mitral regurgitation in dogs (38). We have also shown that mast cell degranulation induced by endothelin-1 or compound 48/80 in the isolated, blood-perfused heart from male rats resulted in MMP activation, extensive collagen matrix degradation, and LV dilatation within 30 min (10, 31). 17β-Estradiol, hereafter referred to as estrogen, has been shown to exert cardioprotective effects in animal studies (35, 37). A few studies have reported direct effects of estrogen on noncardiac mast cells (16, 25), consistent with the observation that mast cells from the aorta of premenopausal women express estrogen receptors (33). Thus the gender differences in the pattern of myocardial remodeling observed in response to an AV fistula may be related to hormonal modulation of mast cell function. Accordingly, we tested the hypothesis that estrogen-mediated effects on cardiac mast cells account for the lack of adverse remodeling in the female heart subjected to chronic volume overload. To this end, we sought to determine whether differences in the response to acute, chemically induced degeneration of cardiac mast cells exist among intact females, ovariectomized females, and ovariectomized females treated with estrogen. Our results indicate that the changes evoked in response to cardiac mast cell degranulation are strongly dependent on the presence of estrogen.

MATERIALS AND METHODS

The surgical procedures and subsequent care of the animals conformed with the principles of the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the protocol was approved by Auburn University’s Institutional Animal Care and Use Committee. In all terminal procedures, the animals were deeply anesthetized using pentobarbital sodium (50 mg/kg ip). To prevent possible effects of a soy-based diet, all animals were maintained on a diet that contained minimal phytoestrogens (Harlan Teklad Diet 2014).

Surgical preparation and experimental protocol. Age-matched adult female Sprague-Dawley rats were randomly divided into groups as follows: intact females (n = 6), ovariectomized females (n = 7), and ovariectomized females treated with estrogen at 0.02 mg·kg⁻¹·day⁻¹ (n = 6). This dose of estrogen was chosen on the basis of preliminary data from our laboratory indicating that ovariectomized females so treated are protected from adverse myocardial remodeling induced by an AV fistula (13). Therefore, females supplemented with estrogen were implanted subcutaneously with 0.25-mg 60-day time-release pellets (Innovative Research of America, Inc., Westerville, OH). The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
The ovariectomy procedure was performed 2 wk before isolated heart function was obtained. Briefly, with the rat under halothane inhalation anesthesia, the ovaries are approached via a ventral abdominal laparotomy, the ovarian pedicle was ligated, and the ovary was excised. Standard techniques were used to close the abdominal musculature and skin with absorbable sutures and autoclips, respectively.

**Experimental design.** The effects of cardiac mast cell degranulation on ventricular function were determined using an isolated, blood-perfused heart preparation as previously described (8). Briefly, the ascending thoracic aorta in the anesthetized rat was cannulated for continuous retrograde perfusion of the heart via an apparatus consisting of a pressurized perfusion reservoir (100–105 mmHg) and a venous collection reservoir connected in circuit with a female support rat. The perfusion apparatus was primed with blood obtained from female blood donors. In the case of the ovariectomized group, the support and blood donor rats were ovariectomized as well. After a compliant balloon was positioned in the LV, pressure-volume relations for each heart were obtained before and 30 min after infusion of the mast cell secretagogue, compound 48/80, as previously described (10). Administration of 1 ml of 0.9% sterile saline containing 7.2 mg of compound 48/80 (Sigma, St. Louis, MO) to the isolated heart was accomplished by introducing the solution into the pressurized perfusion reservoir and allowing the solution to mix with the reservoir blood and perfuse through the beating heart. In a previous study, this dose of compound 48/80 resulted in ~100% cardiac mast cell degranulation (10). The coronary venous blood containing compound 48/80 was not returned to the support rat.

After the functional studies were completed, the atria and great vessels were removed, and the LV (plus septum) and right ventricle were separated and weighed. A complete transmural section of the LV at midventricle was placed in buffered formalin for fixation, and the remainder was minced into ~1-mm cubes, snap frozen in liquid nitrogen, and stored at −80°C. The formalin-fixed tissue was processed for routine histopathology, and 5-μm-thick paraffin-embedded sections were stained with pinocyanol erythrocynate for visualization of cardiac collagen volume fraction (CVF), i.e., the percentage of collagen area relative to total myocardial area, as previously described (10). Portions of the frozen LV tissue were used to determine the wet weight-to-dry weight ratio as a measure of myocardial water content and for Western blot analysis of protein expression. These analyses were performed with the observer blinded as to the source of the tissue sections.

**MMP activity.** MMP activity in cardiac tissue extracts was determined by standard gelatin zymography procedures using an SDS-PAGE matrix containing gelatin (1 mg/ml) (9). All the zymograms had two lytic bands corresponding to standards for the proenzyme (68 kDa) and activated (62 kDa) forms of gelatinase A (MMP-2) and the proenzyme (95 kDa) and activated (82 kDa) forms of gelatinase B (MMP-9; Chemicon International, Temecula, CA). Each gel was run in duplicate, and extract from a single heart was used as a standard on all gels for comparison with results from different gels. The activity of the lytic bands in the other lanes of a gel was expressed as a percentage of this standard’s activity. Once normalized in this fashion, the percent activities from hearts of each group were averaged, with the intact female group set to 100%.

**Western blot analysis.** Expression of tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-3 protein was determined by Western blotting of immunoprecipitated samples. Protein concentration of LV homogenates was determined using bicinchoninic acid reagent (Pierce, Rockford, IL). Equal amounts of protein (20 μg) were immunoprecipitated using the protein A-agarose bead separation method (Sigma) and monoclonal rabbit anti-human TIMP-1 or TIMP-3 antibodies (Research Diagnostic, Flanders, NJ). Appropriate negative controls were performed by substitution of water for the primary antibody before the addition of agarose beads. Equal amounts of supernatant (4 μl) were separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). Membranes were probed with primary antibodies for TIMP-1 and TIMP-3 (1:1,000) for 4 h at room temperature, washed (30 min), and incubated with anti-rabbit secondary antibody conjugated to horseradish peroxidase (Amersham Biosciences, Piscataway, NJ). Immunoreactive bands were visualized using enhanced chemiluminescence reagents (ECL, Amersham Biosciences) exposed to hyperfilm at the linear range of film density. The films were scanned, and densitometric analysis was performed using Un Scan It Software (Silk Scientific, Orem, UT).

**Statistical analysis.** Statistical analyses were performed with Systat 9.0 software (SPSS, Chicago, IL). All grouped data are expressed as means ± SD, unless otherwise noted. Grouped data comparisons were made by one-way analysis of variance, with intergroup comparisons analyzed using Bonferroni’s post hoc test. Statistical significance was taken to be *P* ≤ 0.05. The pressure-volume curves for each heart before and after administration of compound 48/80 were fit to a third-order nonlinear regression (SigmaPlot, SPSS), and the volumes corresponding to 2.5-mmHg pressure increments were determined. The volumes were then averaged to obtain the final pressure-volume relation for each group. Pressure-volume curves were analyzed using a two-factor repeated-measures ANOVA.

**RESULTS**

Histological examination demonstrated comparable, extensive compound 48/80-induced mast cell degranulation in all groups (i.e., >95%). Myocardial water also increased ~3% in the three groups, presumably from mast cell-released histamine. However, in contrast to our findings in a previous study (10), mast cell degranulation did not result in increases in MMP activity in the hearts from intact and estrogen-treated ovariectomized female rats. Only in the ovariectomized females not receiving supplemental estrogen did compound 48/80 cause 133% and 112% elevation in MMP-2 activity above that of intact and estrogen-treated ovariectomized females, respectively (*P* ≤ 0.007; Fig. 1). Average density of the latent MMP-2 band was not different among groups. Although bands representative of latent and active MMP-9 were present, compound 48/80 had no effect on the density of these bands. Moreover, intensity of the MMP-9 bands was markedly less...
than that of the MMP-2 bands. Similarly, there were no differences in TIMP-1 and TIMP-3 expression among the three groups (Fig. 2).

The significant increase in MMP-2 activity in the hearts from ovariectomized females after treatment with compound 48/80 was associated with a significant reduction in CVF below that of intact females (0.46 ± 0.23 vs. 0.73 ± 0.13%, \( P \leq 0.05 \)). Conversely, estrogen replacement in ovariectomized females prevented this significant compound 48/80-induced decrease in CVF (0.60 ± 0.09%, \( P \leq 0.05 \) vs. ovariectomized females; Fig. 3).

Consistent with our previous findings of cardioprotection in intact females, hearts from normal intact females exposed to compound 48/80 did not develop an alteration in the LV pressure-volume curve after mast cell degranulation (Table 1). However, hearts from ovariectomized females developed a significant parallel rightward shift in the pressure-volume relation after exposure to compound 48/80. Estrogen replacement in ovariectomized females effectively prevented a compound 48/80-induced rightward shift in the pressure-volume relation, reproducing the findings in intact females. Concurrent with a significant rightward shift in the pressure-volume curve after compound 48/80 exposure in ovariectomized female hearts, end-diastolic volume at 0 mmHg increased significantly (Fig. 4). However, end-diastolic volume at 0 mmHg was not significantly increased in intact and estrogen-treated ovariectomized female rats. Myocardial compliance, as assessed by the volume required to increase end-diastolic pressure from 0 to 25 mmHg, was not different in any of the groups after exposure to compound 48/80 (Table 1).

**DISCUSSION**

Gender differences in the susceptibility to cardiovascular disease have been established in humans and in animal models (6, 11, 14, 20, 28, 36), but the mechanisms affording this cardioprotection in females are not understood. An increased incidence of cardiovascular disease in postmenopausal women suggests that ovarian hormones may play a role in this cardioprotection (24). Estrogen acts through receptor-mediated pathways and, among other effects, has been shown to inhibit the development of atherosclerosis (2, 19) and increase endothelial and inducible nitric oxide synthase activity (1, 3, 21, 34). However, reduced formation of atherosclerosis and vasodilation cannot fully account for the cardioprotective actions of estrogen. Thus, although numerous possible actions of estrogen

<table>
<thead>
<tr>
<th>Group</th>
<th>( V_0 ) ( \mu l )</th>
<th>( \Delta V_{25} ) ( \mu l )</th>
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<tbody>
<tr>
<td>Intact</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-48/80</td>
<td>292.0±33.6</td>
<td>91.2±34.5</td>
</tr>
<tr>
<td>Post-48/80</td>
<td>303.3±30.3</td>
<td>77.0±27.9</td>
</tr>
<tr>
<td>OX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-48/80</td>
<td>312.8±20.3</td>
<td>46.1±16.5</td>
</tr>
<tr>
<td>Post-48/80</td>
<td>360.2±21.1*#</td>
<td>36.7±12.3</td>
</tr>
<tr>
<td>OX + Est</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-48/80</td>
<td>321.0±14.8</td>
<td>67.5±18.2</td>
</tr>
<tr>
<td>Post-48/80</td>
<td>327.1±11.0</td>
<td>53.0±12.5</td>
</tr>
</tbody>
</table>

Values are means ± SE. OX, ovariectomized; Est, estrogen; \( V_0 \), left ventricular (LV) volume at 0-mmHg end-diastolic pressure; \( \Delta V_{25} \), change in LV volume between 0 and 25 mmHg, end-diastolic pressure. *\( P < 0.05 \) vs intact female. #\( P \leq 0.05 \) vs OX + Est female.
have been examined, this is the first study to evaluate the effect of estrogen on cardiac mast cell-mediated myocardial remodeling.

Because our previous studies demonstrated that cardiac mast cell degranulation mediates MMP activation and extracellular matrix degradation (10, 23, 31), our hypothesis was that cardioprotection in intact females would be due to estrogen-mediated prevention of mast cell degranulation. Accordingly, the purpose of this study was to determine whether gender differences exist in the susceptibility to mast cell degranulation or in the initiation of myocardial remodeling after chemically induced mast cell degranulation. However, because extensive mast cell degranulation was noted in all the female groups stimulated with compound 48/80, mast cell stabilization does not appear to be the mechanism responsible for estrogen-mediated cardioprotection. Although our initial hypothesis was incorrect, this is the first study to demonstrate that estrogen prevents the mast cell-mediated MMP activation and extracellular matrix degradation previously demonstrated in male rats (10, 31). Although this study did not address whether the estrogen cardioprotective effect was receptor mediated, preliminary data from our laboratory indicate the presence of estrogen receptors in mast cells isolated from male and female rat hearts. Therefore, greater insight into mechanisms of action will require studies using estrogen receptor antagonists.

MMP-2 activity in this study was assessed using gelatinase zymography. Although this technique does not directly examine collagenase activity, one would expect the activities of the various MMPs in the collagen degradation cascade to be tightly coupled (32). This was demonstrated by Gunja-Smith et al. (17), who found zymographic gelatinase activity to be closely correlated with collagenase activity determined by tritium-labeled telopeptide-free collagen degradation. Direct evidence demonstrating the ability of cardiac mast cells to activate MMPs in normal male hearts was established by a recent study from our laboratory (10). This study examined the response to chemically induced mast cell degranulation using compound 48/80 and found that cardiac mast cell degranulation resulted in a significant increase in myocardial MMP-2 activity, degradation of the myocardial extracellular matrix, and ventricular dilatation. The findings reported here are consistent with this study in males, in that administration of compound 48/80 to hearts of the ovariectomized female group significantly increased MMP-2 activity relative to that of intact and estrogen-treated ovariectomized females. Because latent MMP-2 levels were similar for all groups, this difference in MMP-2 activity does not appear to be due to estrogen-mediated suppression of MMP expression. Nor can these differences in MMP-2 activity be attributed to estrogen-mediated alterations in TIMP-1 and TIMP-3 expression. Rather, these findings indicate that decreased release of the mast cell enzymes responsible for MMP activation prevented the increase in MMP activity in the intact and estrogen-treated ovariectomized groups. In support of this conclusion is the finding of Harnish et al. (18) that noncardiac mast cell proteases were downregulated by estrogen.

The development of extracellular matrix degradation and ventricular dilatation in the ovariectomized female hearts is consistent with the observed changes in MMP-2 activity. These changes were also comparable to our previous findings demonstrating a parallel rightward shift in the pressure-volume relation in normal male hearts after treatment with compound 48/80 (10). In contrast to the significant LV dilatation and degradation of interstitial collagen (i.e., CVF) in the ovariectomized group, the pressure-volume relations before and after administration of compound 48/80 were unchanged in the hearts from the intact and estrogen-treated ovariectomized rats. Estrogen replacement also effectively prevented the significant decrease in the CVF in the untreated ovariectomized females.

Mast cell-derived histamine is known to increase vascular permeability. Accordingly, it was not surprising that myocardial edema was seen secondary to mast cell degranulation. In all likelihood, this edema was responsible for the nonsignificant trend of increased ventricular stiffness, i.e., reduction in volume required to increase end-diastolic pressure from 0 to 25 mmHg (Table 1), after treatment with compound 48/80. However, the fact that the extent of myocardial edema was comparable in all three groups indicates that histamine did not contribute to the rapidly induced myocardial remodeling. Comparable ventricular dilatation and extracellular matrix degradation independent of histamine were reported within a similar 30-min period by MacKenna et al. (29) in rat hearts perfused with bacterial collagenase.

These differences in MMP-2 activity and extracellular matrix degradation mediated by estrogen suggest an explanation for the different patterns of myocardial remodeling produced in male and female rats in the AV fistula model of heart failure (6, 8, 14). Males develop eccentric hypertrophy, marked ventricular dilatation, and heart failure within 20 wk after a fistula was created (8), whereas intact females develop concentric hypertrophy without ventricular dilatation, and ventricular function is maintained (14). However, in a similar long-term study, ovariectomy eliminated this cardioprotective effect, with an AV fistula producing eccentric hypertrophy and ventricular dilatation comparable to that in males (6). The prevention of ventricular dilatation by estrogen is significant in light of the fact that ventricular dilatation is an independent risk factor in the progression to decompensated heart failure (26).

Although estrogen has been shown to significantly affect MMP activity in the female reproductive system (27, 30), our understanding of the effect of estrogen on cardiac MMPs is limited (41). Xu et al. (41) found that ovariectomy in otherwise normal aged rats resulted in a significant reduction in myocardial MMP-2 protein expression, with this decrease in MMP-2 expression being reversed by estrogen replacement. Conversely, although we saw no effect of ovariectomy on latent MMP-2 expression, we found a significant increase in mast cell-mediated MMP-2 activation in hearts from ovariectomized rats. The reason for these disparate findings is not clear but may be related to differences in age and duration of ovariectomy-induced menopause between the studies.

Estrogen has been shown to have anti-inflammatory effects (18, 25, 39, 40). Thus alterations in mast cell content due to estrogen suggests another possible mechanism. Harnish et al. (18) found that estrogen treatment was associated with a reduction in expression of mast cell proteases in colons of diseased animals and improved intestinal health in a rat model of inflammatory bowel disease. Furthermore, this study demonstrated that estrogen pretreatment of bone marrow-derived mast cells repressed production of several cytokines, including tumor necrosis factor-α (TNF-α), interleukin-6, and interleukin-13. Although all cardiac cell types have the ability to synthesize TNF-α, emerging evidence indicates that constitut-
tive expression of TNF-α is localized in cardiac mast cells (5, 12, 15). In addition, preliminary findings indicate suppression of TNF-α synthesis and/or release by ovarian hormones as the mechanism responsible for the gender-specific cardioprotection from adverse myocardial remodeling secondary to chronic volume overload (7). However, the relationship between TNF-α and MMP activation in modulating myocardial remodeling remains to be elucidated.

In summary, gender differences exist in the response of the isolated heart to compound 48/80-induced mast cell degranulation. Hearts from normal males and ovariectomized females respond to mast cell degranulation with significant increases in active MMP-2 levels, significant reductions in CVF, and a rightward shift in the pressure-volume relation. In contrast, hearts of intact and estrogen-supplemented ovariectomized females do not undergo any of these changes in response to compound 48/80 administration. Although knowledge of the effects of estrogen on mast cells is limited, the results of this and other studies clearly indicate that estrogen can affect the composition and/or release of mast cell contents, which, in turn, affect MMP activation, extracellular matrix degradation, and cardiac remodeling.

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REFERENCES


